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(57) Abstract

The present invention exploits a novel mechanism for photosensitizer localization, namely interaction with the high-affinity receptors which mediate the hormonal signals transmitted by steroids (and some other hormones such as thyroxine, retinoids, and members of vitamin D family). These receptors are expressed only in specific cell types - and by their expression they confer hormone sensitivity on those cells. This invention provides hormone/chromophore conjugates which have reasonable binding affinity towards the hormone receptor protein and methods of administering them to patients as specific photosensitizing agents which can direct lethal damage towards receptor-positive cell lines upon irradiation with visible light. These hormone/chromophore conjugates bound to nuclear hormone receptors can be used as selective molecular delivery systems for photodynamic therapy.

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PHOTODYNAMIC THERAPY USING NUCLEAR HORMONE RECEPTORS TO TARGET PHOTOSENSITIZERS

Background of the Invention

Field of the Invention

This invention is directed to ligand-photosensitizer conjugates and their use in photodynamic therapy. In particular, this invention involves conjugation of photosensitizing agents to hormones which bind to receptors that transport them to intracellular receptors, and to photodynamic therapy of tumors characterized by the presence of these receptors.

Review of Related Art

One of the principal modes of action of pharmaceutical agents is inhibition or outright killing of selected cell types -- either cells of pathogenic organisms or selected cells of the patient her/himself. Cancer chemotherapy represents the major example of the latter procedure, with the objective being to eliminate tumor cells selectively. As is well-known, the degree of selectivity of cancer chemotherapeutic agents varies, but typically is far from perfect, with the result being numerous very unpleasant side effects (*e.g.*, nausea, hair-loss, weakness, anemia, etc.). In fact, the need to restrict these side-effects limits the dosage and duration of most chemotherapeutic anti-cancer regimens.

Photodynamic therapy (PDT) is a relatively new approach to selective cell killing which already has established itself as a useful tool for treating a variety of inoperable cancers (chiefly of the larynx, lung, bladder and face) as well as some forms of skin cancer. The basis for this method is selective uptake of a suitable light-absorbing material into the tumor cells followed by irradiation with visible light. Absorption of light produces excited states of the colored substance(s) which in turn transfer their excitation energy to adjacent oxygen molecules, converting them into singlet-state oxygen, a highly reactive (and therefore quite toxic) chemical species. The colored (chromophoric) material thus acts as a photosensitizer:

in its presence a sufficient dose of visible light kills cells; absent the photosensitizer, the light has no harmful effects.

The great advantage of PDT over other types of anti-tumor therapy lies in its dual selectivity: first a sensitizer is designed to target only certain types of cells, and secondly, the harmful effect will only be expressed when and where visible light is applied. Successful application of a photosensitizer not only depends upon the degree to which it localizes selectively in the desired cell type, but also upon its photophysical properties. For example, a high yield of excited-state triplets and maximum light absorption in the range of 650-800 nm, is preferred.

The FDA has just approved the first (non-specific) PDT agent (PHOTOFRIN™) for routine clinical use. In the case of this archetypal PDT agent (also called hematoporphyrin derivative or HPD), clinicians capitalize on the selective uptake (and retention) of the sensitizer by tumors, waiting for the attainment of an optimal ratio of the sensitizer concentration in tumor cells to the concentration in normal tissues. Then they irradiate the tumor as selectively as possible (often with skillful use of fiber-optic devices), achieving a very highly selective kill of tumor cells with minimal damage to normal cells. Such damage as does occur for normal tissues takes place only in the vicinity of the tumor, in contrast to the systemic side-effects of ordinary chemotherapeutic agents.

Selective targeting of photosensitizers, however, remains a problem area. The localization of HPD in tumors (noted initially by the associated fluorescence) made the first applications of PDT possible; though the basis for the phenomenon still remains poorly understood. HPD has significant limitations, not least of which is skin sensitization (necessitating prolonged protection of patients from sunlight). Subsequent efforts to c ntrol the localization of photosensitizing chromophores have made use of (1) antibody conjugates, (2) micr sphere conjugates, (3) antibody-b und

liposomes, and (4) lipoproteins, especially LDLs. Recently, T.V. Akhlynina, et al., coupled chlorin e_6 to insulin, a hormone that is internalized by cells through endocytosis ("Insulin-mediated Intracellular Targeting Enhances the Photodynamic Activity of Chlorin e-6," (1996), *Cancer Research* 55:1014-1019). They reported that the chlorin e_6/insulin was taken up by cells endocytotically, and that the photodynamic activity of the chlorin e_6/insulin was superior to that of free chlorin e_6. However, there remains a need for additional specifically-targeted photosensitizer compositions.

SUMMARY OF THE INVENTION

It is an object of this invention to provide photosensitizer conjugates which are targeted to particular types of cells.

It is a further object of the invention to provide a method for treating cancer patients whose tumors have cells characterized by the presence of intracellular hormone receptors which respond to presence of cognate hormone by binding and translocation of the hormones to specific nuclear sites. These and other objects are provided in one or more of the following embodiments.

In one embodiment, this invention provides a method for selectively killing cells having a nuclear hormone receptor by photodynamic therapy (PDT), comprising administering to a patient a conjugate comprising a photosensitizer moiety coupled to a ligand moiety which specifically binds the nuclear hormone receptor and illuminating tissue containing the cell with light having a wavelength absorbed by the photosensitizer moiety, preferably a wavelength from about 600 nm to about 850 nm. In general, the ligand moiety is a cognate nuclear hormone of the nuclear hormone receptor, or a derivative or analog thereof exhibiting at least one biological activity associated with binding to the nuclear hormone receptor. The method preferably includes a waiting period after administering the conjugate for a time interval sufficient to allow physiological clearance of the conjugate

from cells and tissues not expressing the nuclear hormone receptor, whereby the concentration of conjugate in the cells having the nuclear hormone receptor is greater than the concentration of conjugate in cells not having the nuclear hormone receptor (i.e., there is a therapeutic ratio having higher concentration in the target cells).

In another embodiment, this invention provides a conjugate comprising a photosensitizer moiety coupled to a ligand which specifically binds to a member of the nuclear hormone receptor superfamily, the coupling preferably being by a covalent linkage, more preferably by a covalent linkage which is uncharged at physiological pH. In a preferred embodiment, the covalent linkage includes from 1 to 10 methylene groups. Preferably, the conjugate according to this invention contains a ligand which is an agonist or antagonist of a receptor of the nuclear hormone receptor superfamily, more preferably, the ligand is an estrogen, androgen, progestin, glucocorticoid, mineralocorticoid, thyroid hormone, retinoid, or member of the vitamin D family. The preferred photosensitizer moiety absorbs light having a wavelength of from about 600 nm to about 850 nm; more preferably, the photosensitizer is selected from the group consisting of chlorins, purpurins, benzoporphyrins, phthalocyanines, and porphines. In general, conjugate according to this invention is membrane-permeable, usually determined either by uptake of the conjugate by mammalian cells *in vitro* or by the conjugate having a permeability coefficient for a lecithin black lipid membrane of at least 10^{-10} cm/sec.

The present invention exploits a novel mechanism for photosensitizer localization, namely interaction with the high-affinity receptors which mediate the hormonal signals transmitted by steroids (and some other hormones such as thyroxine, retinoids, and members of vitamin D family). These receptors are expressed only in specific cell types -- and by their expression they c nfer hormone sensitivity on those cells. This

invention provides hormone/chromophore conjugates which have reasonable binding affinity towards the hormone receptor protein and methods of administering them to patients as specific photosensitizing agents which can direct lethal damage towards receptor-positive cell lines upon irradiation with visible light. These hormone/chromophore conjugates bound to nuclear hormone receptors can be used as selective molecular delivery systems for photodynamic therapy.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 shows the absorption spectrum of chlorin e_6.

Figure 2 shows the molecular structure and absorption spectrum of another well-studied PDT chromophore, benzoporphyrin derivative monoacid (BPD-MA).

Figure 3 shows laser dyes which can be coupled to estrogens in a fashion analogous to that for chlorin e_6 and BPD-MA.

Figures 4a and 4b show reaction schemes for synthesis of ECC-1 and ECC-2, respectively.

Figure 5a details a suitable synthetic scheme for testosterone-chlorin conjugate (TCC-1). This compound targets the chlorin photosensitizer moiety to androgen-receptor (AR)-positive cells.

Figure 5b shows a suitable synthetic scheme for preparation of tamoxifen-chlorin e_6 conjugate.

Figure 5c shows structures of representative photosensitizer conjugates.

Figures 6a and 6b are bar graphs of the dose respose of OVCAR-3 cells to CDME and ECC-1, respectively.

Figures 7a and 7b are line graphs of the dose respose of OVCAR-3 cells to CDME and ECC-1, respectively.

Figures 8a and 8b are bar graphs of the dose respose of MCF-7 cells to CDME and ECC-1, respectively.

Figures 9a and 9b are line graphs of the dose respose of MCF-7cells to CDME and ECC-1, respectively.

Figures 10a and 10b are bar graphs of the dose respose of EJ bladder cells to CDME and ECC-1, respectively.

Figures 11a and 11b are line graphs of the dose respose of EJ bladder cells to CDME and ECC-1, respectively.

Figure 12 shows structures of ECC-2 and various related compounds and their equilibrium constants for binding to human estrogen receptor

DETAILED DESCRIPTION OF THE EMBODIMENTS

The current procedures to eliminate tumor cells are chemotherapy, radiotherapy and surgery. Unfortunately only 50% of the patients who develop malignant tumors are cured by these treatments. Other factors disfavoring cancer chemotherapy are the numerous unpleasant side-effects caused by the chemotherapeutic agents, *e.g.*, nausea, hair-loss, anemia., etc. There is therefore a need for new treatment modalities both for more effective cures and to restrict the side-effects.

Photodynamic therapy (PDT) is a relatively new approach in treatment of cancers and has already established itself as a useful tool in the treatment of certain cancers, for example, lung, bladder and some forms of skin cancer. In PDT, photoactivable compounds, called photosensitizers, are selectively taken up into tumor cells. The photosensitizers are activated (and cause tumor cell killing) when they are irradiated at the appropriate wavelength in the visible light region. Absorption of light produces excited states of the photosensitizers which in turn transfer the excitation energy to adjacent oxygen molecules, converting them into the highly reactive and toxic singlet-state oxygen. Tumor destruction is generally believed to occur via the singlet-state oxygen or by the production of cytotoxic radical species from the ph tosensitizer itself.

PDT has an advantage over other anti-tumor therapies because of its selectivity: (1) sensitizers are designed which only target certain cell types[1] and (2) only those sites are affected where light with the appropriate wavelength is applied. The optimal ratio of sensitizer concentration in tumor cells to the concentration in normal tissues is awaited before irradiating the tumor as selectively as possible. This way a highly selective killing of the tumor cells is achieved with minimal damage to the normal tissues.

The selectivity of any type of PDT depends upon preferentially localizing the photosensitizer in the target tissue or cell type, followed by selective irradiation of the target tissue. A significant unwanted side-effect of PHOTOFRIN (PF) is prolonged cutaneous phototoxicity, necessitating prolonged protection from sunlight. Therefore, photosensitizers with more selective tumor-localizing properties are needed. In attempts to control the localization of photosensitizing chromophores, sensitizers have been bound to antibodies, microspheres, and antibody-bound lipoproteins.

The present invention provides another mechanism to localize photosensitizers, the interaction with the high-affinity receptors which mediate the hormonal signals transmitted by steroids and a variety of other low molecular weight bioactive substances. These receptors are expressed in specific cell types and thus confer hormone sensitivity on those cells. By coupling ligands which bind to these high-affinity receptors to photosensitizers, the nuclear hormone receptors can function as localizing agents to concentrate ligand-photosensitizer conjugates in particular cells. Photodynamic therapy (PDT) involving illumination of these localized

[1] A variety of photosensitizers are being investigated preclinically, both cationic and anionic. Clinically, a mixture of anionic porphyrins, called Hematoporphyrin derivative (HPD) or PHOTOFRIN (PF), has been studied the most extensively.

photosensitizer/ligand conjugates provides enhanced selectivity for the targeted cells/tissues.

Ligands for Targeting Photosensitizers

Conjugates according to this invention are made up of a photosensitizing chromphore coupled to a ligand moiety corresponding to a low-molecular-weight lipophilic compound which is specifically bound by a member of the nuclear receptor gene superfamily. The nuclear hormone receptor "superfamily" includes the proteins specific for response to the following hormones (1) estrogen (estradiol, estriol, and estrone), (2) androgen (testosterone and dihydrotestosterone), (3) progesterone, (4) mineralocorticoid (aldosterone), (5) glucocorticoid (cortisol and cortisone), (6) thyroid hormones, (7) retinoic acid, (8) vitamin D, and (9) ecdysone (insects). To date more than 150 members of the nuclear receptor superfamily have been identified, including the ones just mentioned -- and their isoforms -- as well as a large number of "orphan" receptors (for which there is as yet no known ligand). An extensive list of members of the nuclear hormone receptor superfamily is provided in Mangelodorf, et al., (1995), Cell, 83: 835-839, incorporated herein by reference, and structural characteristics are discussed in Ribeiro, et al. (1995), Ann. Rev. Med., 46: 443-453, also incorporated herein by reference. Orphan receptors of this superfamily are reviewed in Enmark, et al. (1996), *Molec. Endocrinol.*, 10:1293-1307, also incorporated herein by reference. Each receptor has a DNA binding domain and a ligand binding domain. The superfamily is divided into at least two subfamilies: steroid hormone receptors and thyroid hormone receptors, based on the structure of their DNA binding domain; the ligand binding domain is the most highly conserved domain in the nuclear hormone receptor superfamily (Ribeiro, et al., 1995). In a preferred embodiment of this invention, conjugates contain ligand moieties that bind to members of the thyroid hormone receptor subfamily. In an alternative

preferred embodiment, conjugates contemplated by this invention contain ligands which are specifically recognized by one of the steriod hormone receptors, such as estrogen receptor (ER), progesterone receptor (PR), androgen receptor (AR), glucocorticoid receptor (GR), etc.

As presently understood, the mechanism of steroid hormone action consists of (a) secretion of steroid by the source tissue (ovary in the case of estrogen, for example), (b) transport through the plasma (mediated by albumin, transcortin and/or other lipophilic carrier proteins), (c) uptake through the plasma membrane of cells by simple diffusion, (d) non-covalent binding to the cognate receptor (if present) with concomitant activation of the receptor, and finally (e) interaction of the activated hormone/receptor complex with target sequences in nuclear DNA. The hormone receptor proteins normally reside in the cytosol of target cells until their cognate hormone appears, at which point they undergo a conformation change which exposes a nuclear localization signal that causes migration into the nucleus where they bind to appropriate hormone response elements (HRE's) in the control regions of their target genes. In some cases, unliganded receptors may already be present in the nucleus when they bind to the cognate hormone. By use of a suitable photosensitizing moiety (chromophore) attached to the desired hormone in such a way that the receptor still binds the modified hormone and undergoes (1) the conformation change, (2) nuclear localization and (3) the HRE binding process, a cell expressing the receptor can be made very vulnerable to irradiation in the absorption band of the chromophore. The hormone derivative would also, of course, have to be successfully delivered to the target tissue and enter the cells.

While this proposal discusses the steroid hormones and their cognate receptors and specifically focuses on the ER, the invention as described herein encompasses as ligands in the ligand-photosensitizer c njugate all of the vert brate hormones which act via the same general

mechanism. There are striking similarities in the protein structures of all this diverse family of receptors, strongly suggesting a common evolutionary descent, indicating that a methodology developed successfully to exploit any one of them as delivery vehicle for photosensitizer(s) could be transferred with minor modification to the others. Thus this invention contemplates PDT methods using conjugates which specifically bind receptors for (a) estrogens (estradiol, estriol, estrone, and synthetic analogs such as DES and tamoxifen); (b) androgens (testosterone, dihydrotestosterone, and synthetic analogs); (c) progestins (progesterone and synthetic analogs); (d) mineralocorticoids (aldosterone and analogs); (e) glucocorticoids (cortisol, cortisone, and analogs); (f) thyroid hormone; (g) retinoids (retinoic acid and analogs); and (h) vitamin D (cholecalciferol and related compounds). Additionally, the insect hormone ecdysone acts via the same general mechanism and would be potentially useful as a ligand for model studies.

The ligands contemplated by this invention also include analogs of the hormones which bind to receptors of the nuclear hormone receptor superfamily, both synthetic and naturally-occurring analogs. Analogs within the contemplation of this invention are agonists or antagonists of the cognate hormone of the respective nuclear hormone receptor. Agonists and antagonists will exhibit at least one biological activity associated with the nuclear hormone receptor. Biological activity as contemplated herein relies on binding of a ligand for the particular receptor with an affinity that is competitive with the cognate hormone. Binding by an antagonist may simply block the hormone binding site to preclude activation of the receptor by the hormone. Agonists will bind to the receptor in a manner that promotes some or all of the physiological activities associated with hormone action. Photosensitizer conjugates according to this invention will act as agonists or antagonists of the respective nuclear hormone receptor.

In general, agonist or antagonist activity is determined using *in vitro* assays specific for the respective hormones. Usually these *in vitro* assays involve culturing cells known to be responsive to the hormone and measuring characteristic responses of these cells to the particular hormone in culture. When the analog is included in such an assay, the activity measured for a particular hormone concentration will differ from that measured in the absence of the analog. Alternatively, analogs may be assayed by simple binding assays which show an affinity which is competitive with the hormone, either by quantitative comparison or direct competition in the assay. Binding assays based on optical or fluorescent detection of the conjugates of this invention are facilitated by the presence of the photosensitizer. Preferred conjugates will bind to their receptor with an affinity that is within an order of magnitude of the affinity of the cognate hormone.

Of particular importance to cancer therapy is the estrogen receptor (ER) which binds estradiol, estriol, estrone and synthetic estrogen agonists/antagonists such as diethylstilbestrol and tamoxifen. Indeed, specific binding to the ER underlies the mechanism of action for tamoxifen, which is currently one of the chemotherapeutic agents most heavily used to combat advanced (metastasized) breast cancer. Conjugates specific for ER are discussed below as exemplary of the practice of this invention; however, analogous conjugates and PDT methods based on ligands of other members of the nuclear hormone receptor superfamily, which will be readily apparent to the skilled artisan from the descriptions herein, are also within the contemplation of this invention.

Photosensitizer Chromophores

Successful application of a PDT agent depends upon (a) its photophysical (chromophoric) characteristics and (b) the degree to which it localizes selectively in the desired cell type. The desirable photophysical

properties include a high yield of excited-state triplets (which insures efficient generation of singlet oxygen), and maximum light absorption in the range of 650-800 nm (where normal mammalian tissue exhibits maximum transparency). Quite a few photosensitizer compounds possessing these properties have been identified and studied with a view to using them in PDT protocols. (See Amato, (1993) *Science*, **262**: 32-33, and Moan (1990), *J. Photochem. Photobiol.* B, **5**: 521-524, incorporated herein by reference.)

Photosensitizers are substances which absorb light (normally in the visible range) and cause other substances (substrates) to undergo chemical reactions under conditions where they would not react in the absence of the sensitizer. Specifically, the substrates are unaffected by light in the absence of the sensitizer. Traditionally, photosensitizers are considered to act either by a so-called Type I mechanism which involves direct reaction between the sensitizer and the substrate, or by a Type II mechanism in which the excited state of the photosensitizer transfers energy to ambient O_2 molecules, converting them from their ground (triplet) state to an excited (singlet) state which in turn reacts vigorously and almost indiscriminately with surrounding organic molecules. Preferred photosensitizers act via a Type II process, and more preferred photosensitizers are chosen (in part) to have a high quantum yield for singlet oxygen formation (0.7 in the case of chlorin e_6, for example). Photosensitizers of this invention preferably have a quantum yield for singlet oxygen formation of from 0.2-0.9, preferably at least 0.2, more preferably at least 0.4. Photosensitizing chromophores according to this invention will absorb light of wavelengths which enhance both tissue penetration and singlet oxygen formation, usually from about 600-850nm, more preferably from 650-800nm (see Moan, 1990). It will be apparent to the skilled artisan that preferred chromophores will absorb light having a wavelength produced by a laser suitable for use in PDT.

Preferred chromophores include those which have already demonstrated effectiveness in PDT. One such molecule, chlorin e_6, has a useful absorption band at 650 nm (cf. Figure 1) and can be obtained as an ethylene diamine derivative (CMA, Figure 1) which affords an particularly effective tether (when combined with the propionyl group of the chlorin e_6 itself). A variety of approaches to coupling the estrogen to the chromophore may be used, including simple reductive amination with sodium cyanoborohydride.

Figure 2 shows the molecular structure and absorption spectrum of another well-studied PDT chromophore, benzoporphyrin derivative monoacid (BPD-MA). It has the advantage of wavelength of maximum absorption in the red region somewhat longer than chlorin e_6. By converting BPD-MA to an ethylene diamine derivative analogous to CMA, this chromophore may be coupled to estrone in essentially the same fashion as indicated for chlorin e_6. This invention further contemplates use of a variety of different PDT agents with varying pharmacokinetic and/or photophysical properties. For example, Figure 3 shows a selection of Kodak laser dyes which may be coupled to estrogens or other ligands in a fashion analogous to that for chlorin e_6 and BPD-MA.

Particularly suitable chromphores are lipophilic rather than hydrophilic. Lipophilic chromphores have been reported to be more effective sensitizers than hydrophilic chromophore, perhaps because hydrophobicity of the chromphore will facilitate transport of the chromophore through the plasma membrane of the target cell. Such chromophores will ease passage of the conjugate of this invention through the plasma membrane which is particularly important for concentration of the conjugate by binding to intracellular receptors.

Many of the chromophores which have been studied for use in various PDT protocols have properties which make them suitable as the

chromophore (photosensitizer moiety) of the conjugates of this invention. Such chromophores include chlorins, purpurins, benzophorphyrin derivatives, phthalocyanines, porphines, and lipophilic dyes, such as rhodamine (see, *e.g.*, Pass, 1993, pp. 446-447). Selection of suitable chromophores (photosensitizer moieties) meeting the criteria disclosed herein is a routine matter for the skilled artisan.

Conjugation of Ligands and Photosensitizers

For the purpose of this invention, the word "conjugate" is used to describe a molecule which contains a hormone (or ligand) moiety coupled to a photosensitizer moiety such that both moieties retain their intrinsic properties of significance for the intended therapeutic function. Coupling will generally be by covalent linkage, although non-covalent connections may be used, so long as the coupling is as least as stable as the hormone-receptor complex, and the conjugate satisfies the other criteria described herein. The intrinsic properties of significance for PDT include, in the case of the hormone/ligand, high affinity for the targeted nuclear hormone receptor, and in the case of the photosensitizer, high quantum yield for singlet O_2 production at the chosen wavelength of irradiation. It is to be understood that such "conjugates" are well-defined, pure chemical compounds of known structure, which may be contrasted with the mixtures of photosensitizers (HPD, PHOTOFRIN I) which were used in early photodynamic therapy experiments. (However, PDT which uses treatment with two or more specific conjugates simultaneously, as described below, is within the contemplation of this invention.)

Conjugates according to this invention may be designed to use nuclear hormone receptors for targeting agents in PDT, by (a) preparing suitable conjugates between the receptor ligand and the photosensitizing moiety; (b) demonstrating uptake of such an agent into cells; (c) demonstrating preferential uptake of the agent into the desired cell/tissue

type only so as to establish a therapeutic ratio of at least 2:1; (d) showing that irradiation of such cells kills them; and (e) proving that the method works in whole animals. Conjugates preferred for use with targeted laser illumination as discussed below will be concentrated (*e.g.*, by binding to the cognate receptor) to provide therapeutic ratios (concentration in target tissue/concentration in surrounding normal, non-malignant, non-target tissue) of from at least about 2, more preferably at least about 10. Particularly preferred photosensitizer conjugates will be concentrated in the target tissue to give a therapeutic ratio of at least about 25:1 (see Moan, 1990, pages 522 and 523). In the case of the steroid hormones there appears not to be a specific membrane transport system to allow uptake into cells - they seem simply to diffuse through the plasma membrane. That means that a steroid-sensitizer conjugate with suitable solubility characteristics may be expected to be able to follow the same path without confronting any kind of specific permeability barrier.

Choice of conjugate structures can be guided (a) by the known structural basis of biological function for various steroids and analogs, which suggest portions of the hormone or ligand molecule which can be modified for covalent coupling without substantial impairment of nuclear hormone receptor binding, and (b) by inspection and molecular modeling studies using the known structures of the ligand-binding domains (LBDs) of the RXR-α, RAR-γ and thyroid hormone receptors (RXR and RAR refer to retinoic acid-binding receptors), as well as any homologous LBD structures. After many years of attempts, crystallographers have solved the three-dimensional structure of one of the ligand-binding domains of this class of highly homologous proteins, and the long-awaited atomic-resolution structures of the nuclear receptor ligand-binding domains have been determined and published (W. Bourguet, M. Ruff, P. Chambon, H. Gronemeyer & D, Moras (1995) "Crystal Structure of the Ligand-binding Domain of the Human

Nuclear Receptor RXR-α," *Nature*, 375:377-382). This information will be of inestimable value in designing PDT agents which are aimed at the receptors as principal targets. This invention therefore contemplates rational modeling of the specific ligand-binding site for any nuclear hormone receptor to develop superior PDT agents which are conjugates of a suitable photosensitizing agent and a hormone/ligand which binds to a receptor of the nuclear hormone receptor superfamily, the receptor being expressed in cells whose destruction is desired, such as cells in tumor tissue. See Bourguet, et al., 1995; Renoud, et al. (1995), "Crystal structure of the RAR-α Ligand-binding domain bound to all-trans retinoic acid, *Nature*, 378: 681-689; Wagner, et al. (1995), "A structural role for hormone in the thyroid hormone receptor, *Nature*, 378: 690-697; Wiertz, et al. (1996), "A canonical structure for the ligand-binding domain of nuclear receptors," *Nature Struc. Biol.*, 3:87-94, all incorporated herein by reference.

Synthesis methods to produce ligand-photosensitizer conjugates of this invention, designed to bind to the ligand binding site of a particular nuclear hormone receptor, will be routine for the skilled artisan, and any ligand-photosensitizer conjugate prepared by such routine synthesis is within the contemplation of this invention. Preferably, the photosensitizer moiety and the ligand moiety are linked by means of a "tether" (flexible chain), which may be an alkyl chain of 1 to 10 methylene groups. The types of linkages most preferred for this invention are typified by those presented with the prototype compounds (ECC-1, ECC-2a, ECC-2b, and TCC-1). They will be short-to-medium length chains (including 1-10 methylene groups or equivalent) and will be attached to the ligand and sensitizer moieties by neutral (uncharged) functionalities which are easily established such as ether (by Michael addition) and amide (by DCC or similar coupling agent). Ester linkages are also possible, but more likely to be cleaved by enzymatic activity *in vivo*. Incorporati n of some additional, uncharged

polar functional groups in the tether (*e.g.*, hydroxyl, ether, thioether) may be desirable to enhance the uptake rates determined in tissue culture and/or animal studies. Tether structures which facilitate transport of the conjugate in the blood stream (from the site of administration to the target cells and/or for clearance of unbound conjugate from the surrounding tissue) are likewise desirable, so long as uptake of the conjugate into the target cell is not unduly compromised. Of course, the skilled artisan will recognize the importance of testing the ability of the resultant compounds to sensitize killing of cells expressing the cognate receptor upon irradiation, and such testing is contemplated as a routine step in design and synthesis of a ligand/photosensitizer conjugate according to this invention.

Both because it is probably the best-studied nuclear hormone receptor, and because it leads directly to a major clinical application (breast cancer), a preferred targeting receptor is the estrogen receptor in human cells. The estrogen receptor (ER) is a steroid receptor of particular importance in cancer therapy, since it is expressed in many breast and ovarian cancer cell lines. Currently, the synthetic estrogen antagonist tamoxifen is used to treat metastasized breast cancer. In one embodiment of this invention, the specificity of a synthetic estrogen-chlorin conjugate (ECC) is used to treat ER-positive breast and ovarian cancer. Particular conjugates targeted via this receptor will be discussed below. Analogous methods for preparation of similar conjugates targeted to other members of the nuclear hormone receptor superfamily (and their use in PDT by analogous procedures) will be readily apparent to the skilled artisan, as will analogous confirmatory assays to monitor the conjugation procedure.

Synthetic conjugates containing the estrogen moiety may be modified covalently to attach suitable chromophoric groups via a short, flexible "tether". Once prepared, these compounds may be tested against breast-cancer cell lines growing in tissue culture. The test cells will be those

expressing ER, while the controls will be ER-negative cells. Efficacy may be demonstrated using an ER-positive cell line growing in tissue culture. As controls, the ER-negative bladder cancer cell line and the unconjugated chlorin dimethylester (CDME) are tested.

Chromophores may be linked to the estrogen framework at the C-17 position, preferably in the axial (α) orientation, though for some applications enantiomeric purity is not necessarily essential. The choice of the C-17 position comes from consideration of the structures of natural hormones as well as analogs. Testosterone and estradiol, for example, differ in the A ligand specificity at this position -- consistent with the estrogenic activity of estrone which has a carbonyl group (rather than an alcohol) at C-17. It also appears probable that the D-ring portion of the ER binding pocket accommodates the dimethylaminoethyl side chain of tamoxifen or the aziridine group in the ER affinity label ketononestrol aziridine (KNA).

Purification and proof of structure of the compounds can be accomplished by standard methods, usually column chromatography (both conventional and HPLC) followed by NMR and mass spectrometry. The pronounced visible absorptions of the chromophores and the conjugates is an asset both in monitoring purification and confirming structure. Once a suitable estrogen/chromophore conjugate has been synthesized, it may be tested using standard functional assays (*e.g.*, biological activity assays). Demonstrating the feasibility of this molecular delivery method involves good control experiments, and different cell lines, some estrogen-receptor (ER)-positive, others ER-negative, are available, while the parent chromophore (chlorin e_6 dimethyl ester) can be used as a ligand control. MCF-7, a non-metastatic breast cancer line, is available as an experimental cell line (ER -positive), and control cell lines may be selected from MDA-MB-435, a metastatic (ER-negative) breast cancer line, human keratinocytes

(which will also yield information about potential skin phototoxicity), and T4 bladder cancer cells.

ECC-1, a conjugate between an estrogen moiety and chlorin e_6, a porphyrin derivative which acts as a PDT sensitizer, can be used to target cells which contain the estrogen receptor (*e.g.*, most metastatic breast cancer cells). A synthetic procedure for preparing ECC-1 is shown in Figure 4a. Because of its greater bulk and hydrophilicity, ECC-1 is not taken up as well as the parent chromophore CDME or the parent hormone estradiol (see Example 1). Despite these problems, the experiments in Example 1 show that ECC-1 is taken up by mammalian cells in culture, has no apparent toxic effects in the absence of light, and is quite effective as a photosensitizer when irradiated at 664 nm. When compared with the control compound, chlorin e_6 dimethyl ester (CDME), the estrogen conjugate was more effective in sensitizing receptor-positive cells under at least some conditions.

A different conjugate which has an ether linkage in the tether between the steroid and chromophore instead of the secondary amine present in ECC-1 has also been prepared. This new compound (ECC-2) will not be protonated at physiological pH (ECC-1 is protonated under such conditions) and that difference significantly improves its ability to cross the hydrophobic portion of the plasma membrane, thereby increasing the rate of uptake into cells. It may also be expected to improve binding to the estrogen receptor protein. In addition, the synthesis of ECC-2 shown in Figure 4b results in a product which has the carboxyl groups on the porphyrin nucleus present as methyl esters rather than free carboxylate anions (as in the case of ECC-1 at pH 7). This means that ECC-2 will have no ionic charges under physiological conditions, a condition which may be expected to improve its uptake into cells.

The overall reaction scheme for synthesis of ECC-2 is given in Figure 4b. The key intermediate (V) has been repeatedly synthesized in

high yield (>80% for each of the four steps) and its structure confirmed by ^{1}H- and ^{13}C-NMR and mass spectroscopy. The synthesis may be completed by carrying out the coupling and deprotection steps (steps #5 and #6 in Figure 4b). It may be possible (even desirable) to deprotect the phenolic hydroxyl group in the A ring of the estrogen moiety before performing the final coupling reaction. Coupling efficiency may also be improved by changing conditions and by use of different carbodiimide reagents in place of DCC, as well as combinations of other reagents with DCC (*e.g.*, C_6F_5OH). By minor modifications, the scheme in Figure 4b can be adapted to synthesis of a variety of homologs of the main compound, particularly those with a variable ether length (set at three methylene groups in Figure 4b).

Variations in the structure of the conjugate which may modify efficacy are within the skill of the ordinary artisan. Alterations in tether length have already been mentioned. Another variation within the contemplation of the invention is modifying the ligand itself. In the estrogen series, syntheses may be based on estriol and also on synthetic estrogen analogs such as diethylstilbestrol (DES), danazol, and tamoxifen. Similar variations are contemplated for the androgenic compounds.

Testosterone-chlorin conjugate (TCC-1) may be used as a more definitive control, as well as a second family of potential PDT agents. These compounds target the chlorin photosensitizer moiety to androgen-receptor (AR)-positive cells. Figure 5a details a suitable synthetic scheme -- which, as can readily be seen, shares a number of features with the estrogen-conjugate chemistry already described.

TCC-1 is a lead compound as a PDT agent for androgen-receptor (AR)-containing cells, such as those found in virtually all metastatic prostate cancer. (The principal forms of therapy for metastatic prostate cancer at the present time involve anti-androgen treatments such as flutamide (an androgen antagonist), leuprolide (an inhibitor of gonadotropin release),

estrogens or orchiectomy.) In the androgen series, testosterone itself binds fairly weakly to the AR -- it is, in fact, considered in many situations to be a prohormone which must be converted to 5α-dihydrotestosterone in order to have any effect. This conversion is effected *in vivo* by 5α-reductase, and the result is a molecule which binds more tightly to the AR. While 5α-reductase may act on TCC-1, thereby enhancing its affinity for AR, it is preferable to carry the synthesis one step further and chemically reduce TCC-1 to its 5α-dihydro counterpart. An alternative process which produces dihydrotestosterone-chlorin conjugate begins with 5α-dihydrotestosterone and proceeds through analogous synthetic steps.

A synthesis scheme for tamoxifen-chlorin e_6 conjugate is shown in Figure 5b. This scheme shows use of Fmoc-aminocaproate as an activated donor of the tether extension. Structures of other representative photosensitizer conjugates are shown in Figure 5c.

The chemical reaction steps set forth above can be used to couple suitable chromophores to ligands which specifically bind other members of the nuclear hormone receptor superfamily to provide conjugates according to this invention. Selection of suitable ligands and sites on the ligand for attachment of the tether is within the skill of the art. For example, a study by Rink, et al. (1996, *Proc. Natl. Acad. Sci. USA*, 93:15063-15068), concerning targeting of cytotoxins to ER+ cells disclosed an alternative ligand design which they used for targeting cytotoxin to ER+ cells. The results of Rink, et al., with cytotoxin coupled to their ligand also indicate that derivatization of the C-7 position of estradiol does not significantly block binding to the estrogen receptor, and therefore the present invention contemplates coupling of chromophores to the C-7 position to produce photosensitizer conjugates according to this invention. Connecting tether moieties as described above to ligands while preserving the ligand's binding function is facilitated by the available structural information regarding the

ligand-binding domain of the nuclear hormone receptor superfamily (see Bourget, et al. 1995; Renaud, et al., 1995; Wagner, et al. 1995; and Wurtz, et al. 1996). Suitable synthetic procedures may be readily adapted by the skilled artisan from those used to derivatize cognate ligands for affinity purification of the cognate receptors (*e.g.*, vitamin D).

Photodynamic Therapy (PDT)

Photodynamic therapy (PDT) uses photosensitizers to damage or destroy unwanted cells *in situ*, such as PDT to destroy hyperplastic or neoplastic tissues by irradiation with long-wavelength visible light. A variety of procedures and protocols have been described as reviewed by Pass, 1993, JNCI, 85: 443-456; Rosenthal, et al., 1994; Ann. Med., 26: 405-409; and Kessel, 1990, "Photodynamic Therapy of Neoplastic Disease", volumes I and II, CRC Press, Boca Raton, all of which are incorporated herein by reference. PDT methods according to this invention differ primarily through use of particular photosensitizer conjugates targeted to cells containing receptors of the nuclear hormone receptor superfamily. While PDT according to this invention is particularly useful for treatment of tumors, the method may be used in any therapeutic situation for which specific destruction of cells that express receptors of the nuclear hormone receptor superfamily is desirable. Adaptation of PDT protocols to accommodate the particular wavelengths absorbed by the conjugates and to selectively illuminate the target tissues is a routine matter for the skilled clinician.

The ligand/photosensitizer conjugates of this invention are specific for a single type of intracellular receptor, and such ligand/photosensitizer conjugates will be taken up preferentially by cells which express the matching (cognate) receptor. For example, a significant fraction of metastatic breast cancer cells express ER and w uld be expected to assimilate and retain a suitable estrogen-related conjugate; prostate cancer

cells would similarly take up a testosterone-derived conjugate; etc. In a preferred embodiment, this invention contemplates PDT using as an agent an estrogen-chlorin conjugate (*e.g.*, ECC-2) which does not carry ionic charges and may thus readily penetrate cell membranes. Given the nanomolar affinities of receptors for their ligands, this process of selective uptake is highly efficient. Conversely, cells from surrounding normal tissue which lack the corresponding receptor would not become loaded with the hormone-photosensitizer conjugates. After administration of the conjugate (typically i.v.), one can wait for it to clear from the body except for receptor-positive tissues, including tumors, after which the latter can be irradiated with a suitable wavelength of visible laser light guided by fiber optics.

Photosensitizer conjugates according to this invention are preferably formulated in pharmaceutical compositions containing the compound and a pharmaceutically acceptable carrier, such as physiological saline or physiological buffer. The pharmaceutical composition may contain other components so long as the other components do not reduce the effectiveness of the compound according to this invention so much that the therapy is negated. Pharmaceutically acceptable carriers are well known, and one skilled in the pharmaceutical art can easily select carriers suitable for particular routes of administration (Remington's Pharmaceutical Sciences, Mack Publishing Co., Easton, PA, 1985). Compositions or dosage forms for systemic, local or topical may include solutions, lotions, ointments, creams, gels, suppositories, sprays, aerosols, suspensions, dusting powder, impregnated bandages and dressings, liposomes, biodegradable polymers, and artificial skin. Typical pharmaceutical carriers used in compositions for local or topical application include alginates, carboxymethylcellulose, methylcellulose, agarose, pectins, gelatins, collagen, vegetable oils, mineral oils, stearic acid, stearyl alcohol,

petrolatum, polyethylene glycol, polysorbate, polylactate, polyglycolate, polyanhydrides, phospholipids, polyvinylpyrrolidone, and the like.

A preferred strategy is to administer these conjugates locally or topically in gels, ointments, solutions, or liposomes. Liposomes containing photosensitizer conjugates according to this invention may be prepared by any of the methods known in the art for preparation of liposomes containing inclusions. Liposomes that are particularly suited for aerosol application to the lungs are described in International Patent Publication WO 93/12756, pages 25-29, incorporated herein by reference.

The concentrations of the photosensitizer conjugate in pharmaceutically acceptable carriers will typically range from 1 μM to 0.1 M. The dose used in a particular formulation or application will be determined by the requirements of the particular type of disease and the constraints imposed by the characteristics and capacities of the carrier materials. Dose administered will depend on a variety of factors, including disease type, patient age, patient weight, and tolerance of toxicity.

Dose will generally be chosen to achieve local peak concentrations in target cells of from 0.01 nM to 10 μM, preferably from 0.1 nM to 100 nM. Peak concentration is the maximum level of photosensitizer in cells expressing the receptor which binds the ligand moiety of the photosensitizer, counting both bound and unbound photosensitizer, following administration of the photosensitizer conjugate. Binding of the ligand moiety by the target receptor will result in higher total amounts of photosensitizer conjugate in the target cell than in neighboring cells or surrounding medium at equilibrium; serum concentrations and/or concentrations in surrounding non-targeted cells will generally be at least 2-fold lower than the concentration in the target cells, preferably at least 5-fold lower, and more preferably at least 10-fold l wer. However, the instantaneous level of photosensitizer in any particular cell will reflect both

systemic pharmacokinetics and local kinetics for transport in and out of cells. Initial dose levels may be selected based on their ability to achieve ambient concentrations shown to be effective in *in vitro* models, such as that used to determine therapeutic index, and *in vivo* models and in clinical trials, up to maximum tolerated levels. The dose of a particular photosensitizer conjugate and duration of therapy for a particular patient can be determined by the skilled clinician using standard pharmacological approaches in view of the present disclosure. The PDT may be monitored by analysis from time to time of blood or body fluid levels of the photosensitizer conjugate according to this invention. The skilled clinician will adjust the dose and duration of therapy based on these measurements.

Pharmaceutical compositions containing any of the photosensitizer conjugates of this invention may be administered by parenteral (subcutaneously, intramuscularly, intravenously, intraperitoneally, intrapleurally, intravesicularly or intrathecally), topical, oral, rectal, or nasal route, at the discretion of the clinician. Lesions in externally accessible surfaces may be treated by non-invasive administration of a photosensitizer according to this invention. In a preferred mode, the compound according to this invention is formulated in a pharmaceutical composition and applied to an externally accessible surface of a patient having a lesion in an externally accessible surface. Externally accessible surfaces include all surfaces that may be reached by non-invasive means (without cutting or puncturing the skin), including the skin surface itself, mucus membranes, such as those covering nasal, oral, gastrointestinal, or urogenital surfaces, and pulmonary surfaces, such as the alveolar sacs. Non-invasive administration includes (1) topical application to the skin in a formulation, such as an ointment or cream, which will retain the compound in a localized area; (2) oral administration; (3) nasal administration as an aerosol; (4)

intravaginal application of the compound formulated in a suppository, cream or foam; (5) rectal administration via suppository, irrigation or other suitable means; (6) bladder irrigation; and (7) administration of aerosolized formulation of the compound to the lung. Aerosolization may be accomplished by well known means, such as the means described in International Patent Publication WO 93/12756, pages 30-32, incorporated herein by reference.

Preferably, clinical protocols for the use of PDT agents according to this invention parallel those already in use with PHOTOFRIN and similar agents (as given full FDA approval in December 1995). The PDT agents will usually be stored at low temperature (preferably frozen) in the dark under an inert atmosphere so as to minimize photooxidation and other degradative processes. Suitable aliquots will be thawed just prior to administration. Typically, treatment will begin with administration of the agent as a sterile solution i.v. Doses may be optimized by standard procedures for the particular type of neoplastic or hyperplastic tissue. Such optimization is a routine matter for the clinical oncologist. PHOTOFRIN doses have typically fallen into the 1-2 mg/kg range; it is contemplated that the conjugates described in this invention will be used at considerably lower levels because of their property of specific binding to target-cell components.

Once the conjugate is infused into the patient, specific binding to the cognate receptors in the cytoplasm of the cells expressing the particular nuclear hormone receptors will concentrate the photosensitizer moiety in the target tissue. Illumination of the target tissue will generally be delayed for a suitable period to permit physiological clearance for any unbound conjugate from cells and tissues not expressing the receptor. Clearance can be monitored by measuring the concentration of the conjugate in peripheral blood or urine, usually by ptical or flu rescent detection of

the photosensitizer moiety. Specificity of PDT is imparted by the favorable concentration ratio of sensitizer in target tissue to sensitizer in non-target cells. In the case of each type of target tissue the time delay between administration of the PDT agent and irradiation with long-wave-length visible light can be established by suitable analytical methods, leading to procedures that can be routinely followed in all cases of the same type. Generally the optimum therapeutic ratio (concentration of drug in target tissue/concentration in normal, non-malignant, non-target tissue) will be obtained within 2-3 days of administration. During this time (and also for as much 4-6 weeks after administration) there may well be some cutaneous photosensitivity and it is preferable for patients to avoid excessive exposure to sunlight and other intense light sources so as to minimize erythremia.

Irradiation of the tissue containing the PDT agent, should preferably take place during the interval of maximum therapeutic ratio. Selection of suitable light sources is discussed in Pass, 1993, page 445, incorporated herein by reference. Depending upon the sensitizer and target, one of several procedures might be followed. If the target is a solid tumor accessible to fiber-optic devices, such devices (with a suitable diffuser on the distal end) can be embedded in the tumor (up to a depth of several centimeters). Light from a tunable dye laser (or possibly a diode laser) could be directed into the fiber and thus to the sensitized tumor site. The wavelength of the light would be chosen to match the absorption bands of the PDT chromophore (*e.g.*, 665-675 nm in the case of chlorin e_6). Different procedures would be followed in the case of target tissues on or close to the skin surface, as in melanoma, psoriasis, etc. In these cases the configuration of the laser and/or fiber-optic light-delivery system would take into account the flat geometry of the target. Irradiation periods can be chosen to deliver to total dose comparable to that currently employed with PHOTOFRIN, viz.

50-300 joules/cm^2, usually administered over a time interval of 5-45 minutes, with 8-20 minutes being typical.

Routine clean-up procedures should be employed as a follow-up to the PDT treatment. For example, a bronchoscope, sigmoscope or other suitable device can be used to trim and remove damaged or necrotic tissue remaining after 24 hours or so post-treatment.

The photosensitizer will be further concentrated in the target cell if it is conjugated to ligands which bind to more than one receptor in the target cell. This may occur in instances where the cell, in addition to expressing the cognate nuclear hormone receptor, also expresses ancillary enzymes which bind to the conjugates, thereby increasing their intracellular concentration still more. In a particular examples, ER-positive cells have the enzyme 17β-hydroxysteroid dehydrogenase, and AR (androgen receptor) containing cells have 5α-reductase, the enzyme responsible for converting testosterone into dihydrotestosterone. Binding of photosensitizer conjugates containing the respective ligands to these enzymes will lead to accumulation of additional photosensitizer in the respective cells.

Alternatively, target cells may express more than one member of the nuclear hormone receptor superfamily, and the enhancement may be provided by using a combination of more than one photosensitizer conjugate in a cocktail. In the case of breast-cancer cells, the vitamin D receptor is also present, hence applying two conjugates simultaneously should be quite feasible, and that would significantly increase the therapeutic ratio (photosensitizer concentration in target cell divided by concentration in adjacent normal tissue). preferably, both photosensitizer conjugates in such a cocktail would absorb light at the same wavelength, which may be accomplished by using conjugates of the same chromophore to different ligands. Synthesis of the corresponding vitamin-D conjugates of chlorin e_6 are described above. Selection f suitable ph tosensitizer conjugates and

application in a cocktail or in multiple substantially contemporaneous administrations is within the skill of the art in view of the present disclosure.

EXAMPLES

In order to facilitate a more complete understanding of the invention, a number of Examples are provided below. However, the scope of the invention is not limited to specific embodiments disclosed in these Examples, which are for purposes of illustration only.

EXAMPLE 1 Photodynamic Effects on Cancer Cell Lines

MCF-7 breast cancer cells, EJ bladder cancer cells and OVCAR-3, an ovarian cancer cell line, were used. The MCF-7's and OVCAR-3's are estrogen-receptor positive; the EJ cells are estrogen-receptor negative. The breast cancer cell line MCF-7 and the OVCAR-3 cells were maintained in Dulbecco's Modified Eagle Media (DMEM) and RPMI, respectively, supplemented with heat-inactivated fetal bovine serum (10% v/v) and gentamicin (0.05 mg/mL) at 37°C, 5% CO_2. The EJ cells were maintained in McCoy's 5A supplemented with heat inactivated fetal bovine serum (5% v/v) and gentamicin (0.05 mg/mL) at 37°C, 5% CO_2. To induce the estrogen receptors, 5μg insulin/mL and 1 nM estradiol was added to the media. ECC-1 was prepared according to the synthetic protocol shown in Figure 4a.

CDME/ECC-1 Uptake Measurements:

The day before incubation, 1.5-2.0x10^5 cancer cells were plated out in a 35 mm petri dish with 2mL medium and placed in an incubator at 37°C and 5% CO_2. The tumor cells were incubated with 0.1, 0.25 and 0.5 μM CDME and ECC-1 for 1 hour. Following incubation, the tumor cells were harvested with Trypsin + EDTA and lysed in 3 mL 0.1N NaOH to extract the CDME and ECC-1. 1 mL of the extract was used for protein determination whereas the other 2 mL were used for measuring CDME and ECC-1 concentrations. 20μl SDS (20%) was added to the

samples to obtain a final concentration of 0.1% SDS. CDME and ECC-1 concentrations were measured on a fluorescence spectrophotometer by exciting at 410 nm and measuring the emission at 664 nm. The CDME and ECC-1 concentrations were calculated by using standard curves and by correcting them for the protein content of each sample. The protein assay was performed using the Biorad protein assay kit.

By means of fluorescence microscopy it could be observed that ECC-1 penetrated the cells and became concentrated in some regions, such as lysosomes in preference to others. In all three cell lines ECC-1 showed no toxicity in the absence of light, even at the highest concentration tested (0.5 μM). In this regard it was completely comparable to the control compound (CDME).

Since photosensitized cell killing (see below) amounts to an indirect measure of uptake of the sensitizer, the survival data can also be used to estimate relative uptake. For example, considering the data for CDME (which should be independent of the expression or lack of expression of the estrogen receptor), the OVCAR-3 (ovarian cancer) cells appear more permeable than the EJ (bladder cancer) cells, since the photosensitized killing in the later case is significantly less, especially at fluences of 1, 5 and 10 J/cm^2 at a dye concentration of 0.1 μM (cf. Figs. 6a & 7a vs. Figs. 10a & 11a). This analysis depends upon the assumption that the intrinsic sensitivity of all three cell types to photosensitized killing is the same. To the extent to which that assumption holds, the CDME data allow the permeability of the cells to be ranked as: OVCAR-3>EJ>MCF-7. For the purpose of this discussion, it will be assumed that the same order holds for ECC-1.

Cytotoxicity Studies:

The key test of the concept that estrogen-conjugated photosensitizers can be targeted to estrogen receptor-positive cells must

come from comparison of the efficiency of cell killing by ECC-1 with that by the control compound CDME (which, incidentally, was the source of the chlorin e_6 moiety used in the synthesis of ECC-1).

1.5-2.0x10^5 tumor cells were plated out in 35 mm dishes with 2 mL medium. The tumor cells were incubated with the photosensitizer and irradiated the next day. The concentration of CDME and ECC in the media was checked with a spectrophotometer (Hewlett Packard) using the extinction coefficient of 20,000 L/mole-cm at 664 nm. After the incubation with CDME and ECC the tumor cells were washed with PBS and maintained in PBS during irradiation with the argon pumped dye laser. A dye laser, containing DCM dye was pumped by the 514.5nm emission of a continuous wave argon ion laser. The dye laser emission was tuned to 664nm, the characteristic wavelength of the photosensitizer, and passed through a 1mm diameter quartz fiber optic. The light beam was focused with lenses onto the sample in a 35mm petri dish. After the PDT treatment the PBS was replaced for medium, and the cells were incubated for 24 hours, after which the cell survival was measured (described below). All experiments and controls were performed on a minimum of three dishes and were done in duplicate.

Cell Survival:

The cell survival was determined by the dimethylthiazol-diphenyltetrazolium bromide (MTT) assay. The tumor cells were incubated in 1.25 mg/mL MTT in DMEM (FCS 10%v/v) without phenol red for 4 hours. The MTT solution was replaced by DMSO (100%) to dissolve the tumor cells and blue crystals prior to reading the optical density (OD) at 577 nm by using the ELISA reader. A dead control, consisting of cells incubated at -18°C, was used as background value and the cell survival was expressed as percentage of the c ntrol. The controls consisted of plates xposed to

neither photosensitizer or light and photosensitizer without light (dark controls) and the protocol was the same as described for the treated samples.

First, consider the results with EJ bladder cancer cells which do not express the estrogen receptor. In all but two cases (cf. Figs. 10 & 11), cell survival was slightly better for the ECC-1-treated cells than for the controls. (The exceptions were for 0.1 μM dye concentration at fluences of 5 and 10 J/cm^2.) This is consistent with a slightly reduced uptake of the ECC-1, which would be expected on the basis of its greater size and additional zwitterionic charge. The differences, however, were small in all cases and fell within the overlapping error bars of the measurements.

For the OVCAR-3 ovarian cancer cells (cf. Figs. 6 & 7), out of the twelve conditions tested with illumination, four showed more effective killing by ECC-1 than by CDME and two of these (0.25 μM dye at fluences of 5 and 10 J/cm^2) corresponded to differences outside the error range. Seven conditions showed greater killing by CDME, but all these differences fell within the error bars. Under one condition the killing was approximately the same for both ECC-1 and CDME. Assuming that uptake of CDME is greater than ECC-1 (as suggested by the EJ cell results described above), there is a distinct indication of a positive result for these ER-containing cells. Further, comparing the ECC-1 killing of OVCAR-3 cells with that of EJ cells (cf. Figs. 6b & 7b vs. Figs. 10b & 11b), the lowest dye concentration (0.1 μM) showed increased killing of the OVCAR-3 cells at all fluences tested (4 conditions) and the medium concentration (0.25 μM) showed increased killing at the two lower fluences (0.5 and 1.0 J/cm^2). At the highest dye concentration (0.5 μM), however, the preferential killing was reversed; ECC-1-treated EJ cells were killed to a greater extent than OVCAR-3 cells at all four fluences. The remaining two cases showed equal extents of killing. These comparisons between ECC-1 effects on the two cell

types involve small differences, mostly within the error limits, but demonstrate trends.

Turning to the MCF-7 breast cancer cell data, comparing the killing sensitized by ECC-1 with that by CDME (Figs. 8 & 9), the ECC-1 proved more effective in six out of the twelve illuminated conditions. At a concentration of 0.25 μM it was superior at every fluence level tested. Under four conditions, the killing by the two dyes was essentially identical, and in the remaining conditions (0.5 μM at fluences of 5 and 10 J/cm^2) CDME killed more effectively than ECC-1. These results demonstrate the targeting of photosensitizer by hormone, especially if one considers that the MCF-7 cells may be the least permeable of the three types to compounds like CDME and ECC-1, with the latter suffering the greater disadvantage from this factor (see discussion above). The differential permeability factor may also explain the fact that ECC-1 killed EJ bladder cells (estrogen receptor-negative, Figs. 10b & 11b) somewhat better than MCF-7 cells (estrogen receptor-positive, Figs. 8b & 9b). Consistent with this explanation is the fact that this differential killing mainly showed up at the highest dye concentration -- where simple mass action effects would be maximum. That contrasts with the instances mentioned above where ECC-1 is clearly better as a photosensitizer than CDME -- all of which involved either the medium or lowest dye concentration conditions (where a favorable interaction with the intracellular receptor would be expected to confer the greatest advantage).

Biological assays are inherently difficult to interpret because of the sensitivity of cells to numerous subtle factors. However, these experiments demonstrated unequivocally that ECC-1 is not toxic to the tested cell lines in the absence of light and that it does act efficiently as a lethal photosensitizer. At least two factors (in addition to any specific shortcomings of the ECC-1 molecule itself) may limit the selectivity

observed here. One has to do with the levels of expression of the estrogen receptor in tissue-culture cells. These may be down-regulated in the absence of suitable stimulatory factors. According to workers in the field, MCF-7 cells fall into this category. Secondly, any endogenous estrogen or estrogen-mimetic compounds would competitively inhibit the binding of ECC-1 to the receptor active site. Growth medium components such as fetal calf serum may contain such compounds, and it has also been recently noted that items of laboratory equipment such as plastic petri dishes sometimes contain detectable amounts of estrogenic substances *[J. Amer. Med. Assn.* 271, 414 (1994)].

EXAMPLE 2 Synthesis of an uncharged conjugate

Some of the features of ECC-1 make it a less-than-ideal candidate for targeting steroid hormone receptors. For one thing, it carries two ionic charges at physiological pH (a plus charge on the protonated secondary amine of the tether, where it is attached to C-17 of the steroid moiety, and a negatively charged carboxylate on one of the side groups of the chlorin ring system). On the basis of the well-known inhibitory effects of charges on the permeability of molecules through lipid bilayers (and cell membranes), this feature is undesirable. (By contrast, CDME, the control compound, has only one anionic carboxylate group *in vivo*.) A second concern is the possibility of stearic or other disruptive effects which would diminish the affinity of the conjugate for the estrogen receptor protein. ECC-1 has comparatively short tether (which includes the positive charge on the secondary amine already discussed) and this may not allow optimum drug/protein binding.

In order to improve upon these features of ECC-1, a second estrogen-chlorin conjugate (ECC-2) may be synthesized by the route outlined in Figure 4b. Starting with estrone (I), the phenol is protected with a benzyl group, then the C-17 keto gr up is reduced with $LiAlH_4$ to give 3-

(O)-benzyl-estra-17β-ol (III). This reacts with acrylonitrile to yield 3-(O)-benzyl-estra-17β-(O)-propionitrile (IV), which, after reduction and deprotection, yields 3-(O)-benzyl-estra-17β-(O)-(3-aminopropyl)estra-3-ol (V). DCC coupling of V with chlorin e_6 dimethyl ester (VI) completes the synthesis of "estrogen-chlorin conjugate #2 (ECC-2)" (VII). This molecule contains the estradiol moiety coupled to the porphyrin nucleus by a 6-atom linker and may be expected to be recognized by the estrogen receptor -- as inferred from the behavior of various other steroids and steroid analogs. The secondary amine linkage (with its positive charge) is replaced by an ether linkage (which will remain neutral under all pH conditions). Furthermore, by using a DCC coupling step at the end, the steroid moiety is reacted with the free carboxyl group of CDME, leaving the other two carboxyls of the porphyrin both in the form of unaltered methyl esters. This will produce a molecule with no ionic charges whatsoever, thereby enhancing permeability through the plasma membranes of target cells. A further feature of the synthesis scheme given in Figure 4b is that it is readily adaptable to tethers of variable length: by choosing diols with n = 0-3, conjugates can be made with 2-5 methylene (i.e., $-CH_2-$) groups in the tether. This will provide flexibility to optimize the conjugate/receptor interaction.

It is also possible to alter the character of the chromophore by using benzporphyrin derivative monoacid (BPD-MA). BPD-MA has even better spectral properties for use in photodynamic therapy than does chlorin e_6. It also had the advantage of being more lipophilic. Since it contains a single free carboxyl group, it is ideally suited to the DCC coupling step envisaged for ECC-2 synthesis (Figure 4b).

Binding of ECC-2 and various related compounds to human estrogen receptor was examined by conpetitive binding studies. Structures and inhibition constants for the compounds tested are shown in Figure 12.

EXAMPLE 3 Synthesis of an androgen-based PDT agent

By using testosterone as the ligand and chlorin e_6 as the photosensitizer, an organic compound was designed to bind to the nuclear testosterone receptor. The main synthetic steps are the construction of the C3 hydroxy on the A ring, the chain elongation at C17 in a Michael addition, followed by reduction of the chain nitrile group into an amine. The final product is obtained by coupling the testosterone derivative to chlorin e_6. The compound may be used in photodynamic therapy (PTD) of cancer cells which are testosterone-responsive, targeting specific cell types and allowing them to be killed by irradiation with visible light. The compound is selectively taken up in cancer cells, and by irradiating with visible light, toxic, reactive singlet oxygen can be produced, resulting in cell destruction. This synthesis produces a molecule capable of targeting testosterone responsive cells and potentially useful in the treatment of metastatic prostate cancer.

For purposes of clarity of understanding, the foregoing invention has been described in some detail by way of illustration and example in conjunction with specific embodiments, although other aspects, advantages and modifications will be apparent to those skilled in the art to which the invention pertains. The foregoing description and examples are intended to illustrate, but not limit the scope of the invention. Modifications of the above-described modes for carrying out the invention that are apparent to persons of skill in medicine, oncology, organic chemistry, pharmacology, and/or related fields are intended to be within the scope of the invention, which is limited only by the appended claims.

All publications and patent applications mentioned in this specification are indicative of the level of skill of those skilled in the art to which this invention pertains. All publications and patent applications are herein incorporated by reference to the same extent as if each individual

publication or patent application was specifically and individually indicated to be incorporated by reference.

CLAIMS:

1. A method for killing cells having a nuclear hormone receptor, comprising:

administering to a patient a conjugate comprising a photosensitizer moiety coupled to a ligand which specifically binds the nuclear hormone receptor; and

illuminating tissue containing the cell with light having a wavelength, light of said wavelength being absorbed by said photosensitizer moiety.

2. The method of claim 1, wherein the ligand is a cognate nuclear hormone of the nuclear hormone receptor, or a derivative or analog thereof exhibiting at least one biological activity associated with binding to the nuclear hormone receptor.

3. The method of claim 1, further comprising waiting after administering the conjugate for a time interval sufficient to allow physiological clearance of the conjugate from cells and tissues not expressing the nuclear hormone receptor, whereby the concentration of conjugate in the cells having the nuclear hormone receptor is greater than the concentration of conjugate in cells not having the nuclear hormone receptor providing a therapeutic ratio.

4. The method of claim 3, wherein the therapeutic ratio is at least 2.

5. The method of claim 1, wherein the wavelength is from about 600 nm to about 850 nm.

6. The method of claim 1, further comprising

administering to the patient a second conjugate comprising a second photosensitizer moiety coupled to a second ligand which specifically binds a second nuclear hormone receptor; said second nuclear hormone receptor being expressed in the cell.

7. The method of claim 6, wherein the second photosensitizer absorbs light of said wavelength.

8. The method of claim 6, wherein both conjugates comprise the same photosensitizer moiety.

9. A conjugate comprising a photosensitizer coupled to a ligand which specifically binds to member of the nuclear hormone receptor superfamily.

10. The conjugate of claim 9, wherein the photosensitizer is coupled to the ligand by a covalent linkage.

11. The conjugate of claim 10, wherein the covalent linkage is uncharged at physiological pH.

12. The conjugate of claim 10, wherein the covalent linkage includes from 1 to 10 methylene groups.

13. The conjugate of claim 9, further wherein the conjugate is membrane-permeable, as determined by uptake of the conjugate by mammalian cells *in vitro*.

14. The conjugate of claim 9, wherein the conjugate has a permeability coefficient for a lecithin black lipid membrane of at least 10^{-10} cm/sec.

15. The conjugate of claim 9, wherein the ligand is an agonist or antagonist of a receptor of the nuclear hormone receptor superfamily.

16. The conjugate of claim 15, wherein the ligand is selected from the group consisting of estrogens, androgens, progestins, glucocorticoids, mineralocorticoids, thyroid hormones, retinoids, and members of the vitamin D family.

17. The conjugate of claim 9, wherein the photosensitizer absorbs light having a wavelength of from about 600 nm to about 850 nm.

18. The conjugate of claim 17, wherein the photosensitizer is selected from the group consisting of purpurins, benzoporphyrins, phthalocyanines, and porphines.

19. The conjugate of claim 17, wherein the photosensitizer is chlorin e_6.

20. A kit containing:

a first photosensitizer coupled to a first ligand which specifically binds to a first member of the nuclear hormone receptor superfamily, and

a second photosensitizer coupled to a second ligand which specifically binds to a second member of the nuclear hormone receptor superfamily.

FIG. 1

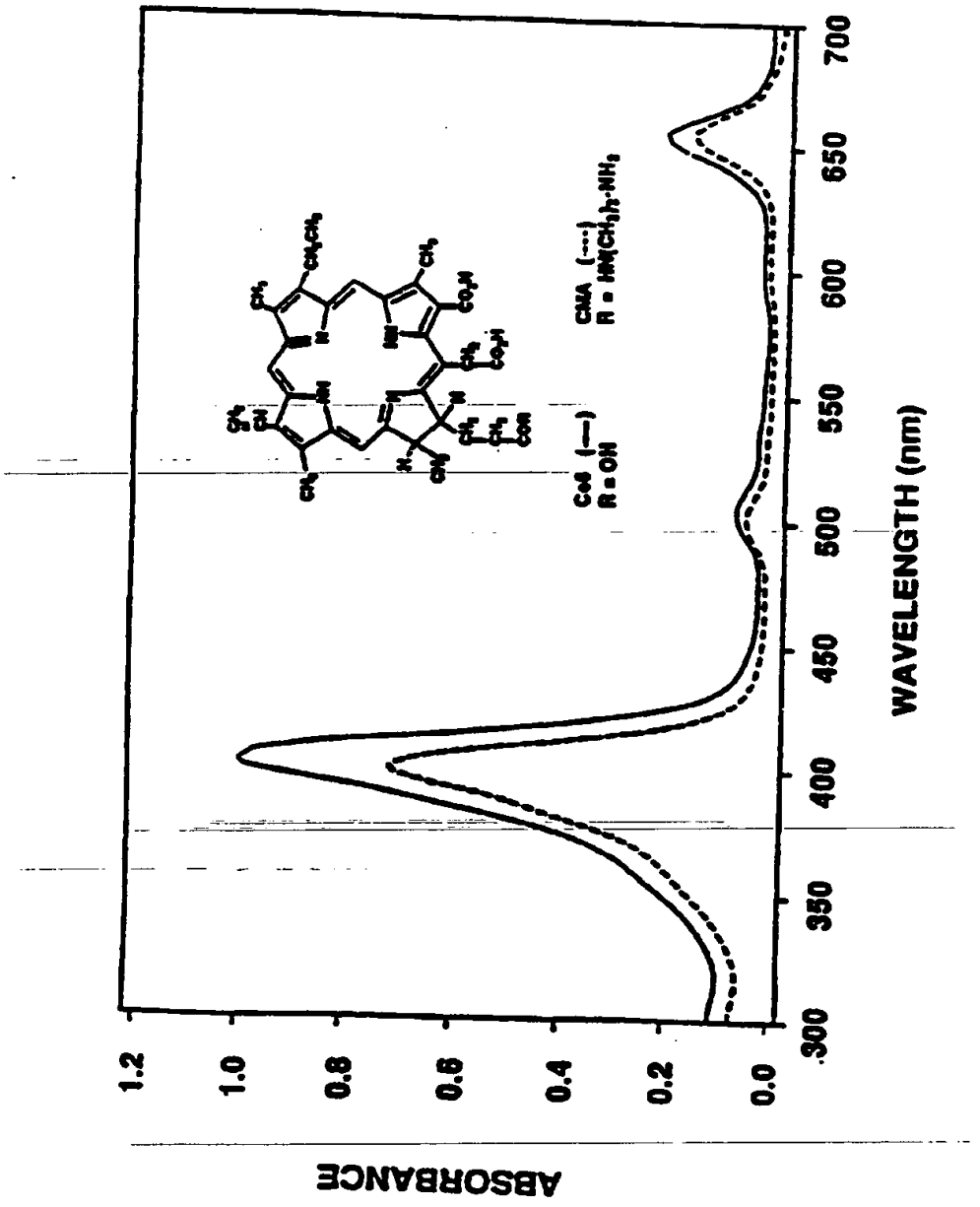

ABSORBANCE
1.2
1.0
0.8
0.6
0.4
0.2
0.0
300
350
400
450
500
550
600
650
700
WAVELENGTH (nm)
CoA (---)
R = HN(CH2)3-NH2
CoE (—)
R = OH

FIG. 2

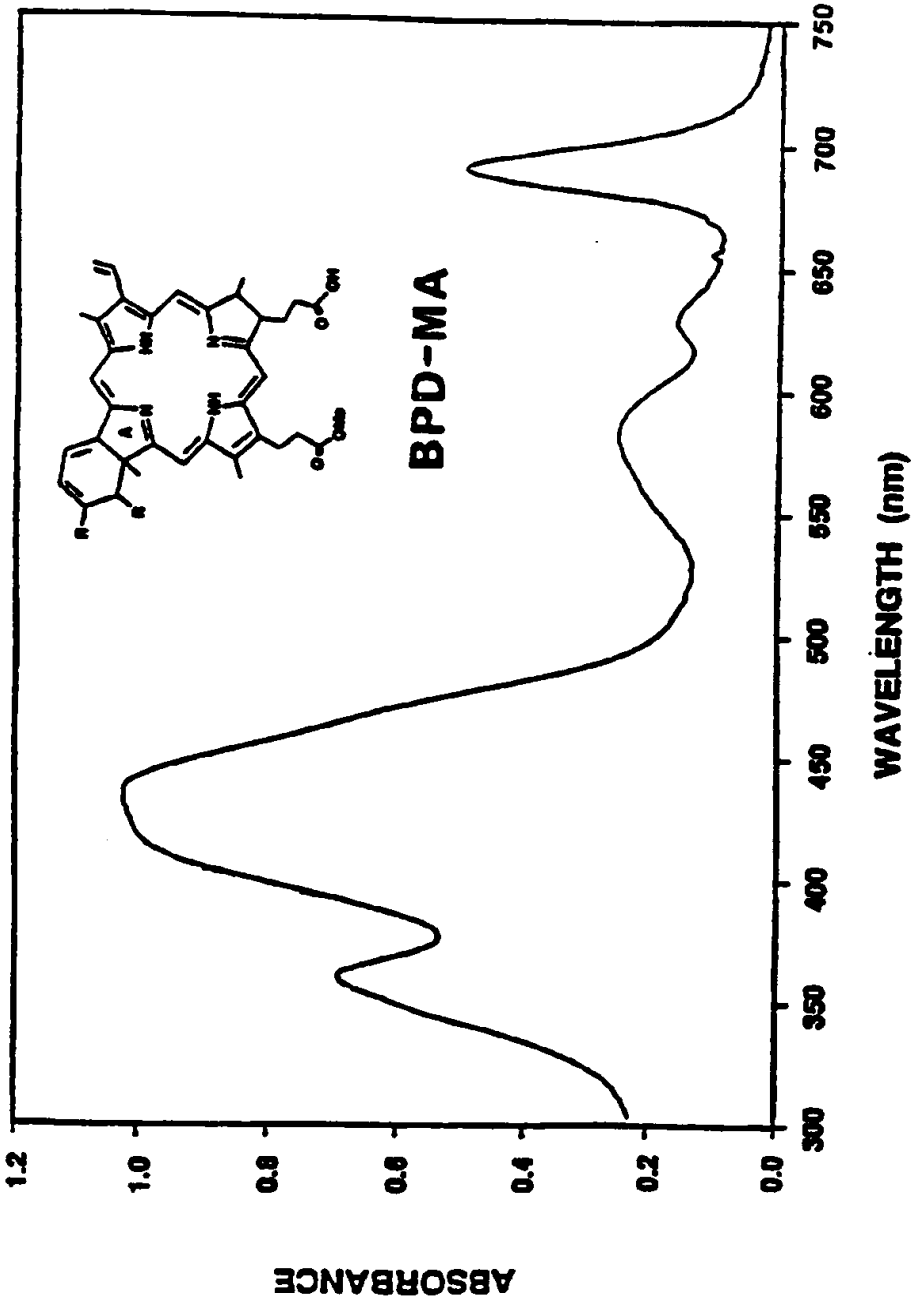
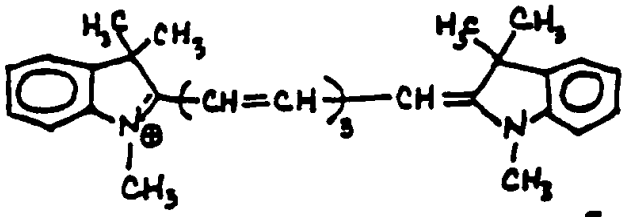
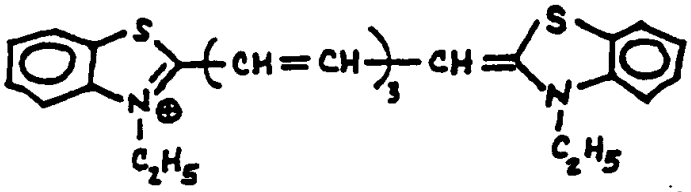

BPD-MA
ABSORBANCE
1.2
1.0
0.8
0.6
0.4
0.2
0.0
300
350
400
450
500
550
600
650
700
750
WAVELENGTH (nm)

FIG 3



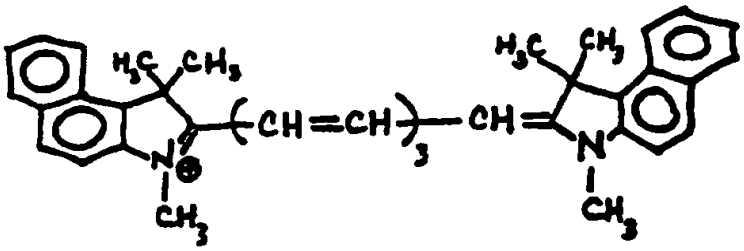
1,1',3,3,3',3'-hexamethyl-indotricarbocyanine (HITC) [as perchlorate or iodide]

λ_{max} = 750 nm ϵ_{max} = 1.9×10^5 L/mol·cm



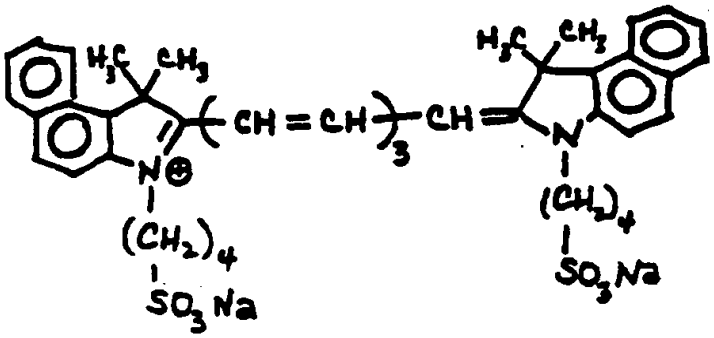
3,3'-diethylthiatricarbocyanine (DTTC) [as perchlorate or iodide]

λ_{max} = 772 nm ϵ_{max} = 1.65×10^5 L/mol·cm



1,1',3,3,3',3'-hexamethyl 4,4',5,5'-dibenzo-2,2'-indotricarbocyanine (HDITCP) [as perchlorate]

λ_{max} = 780 nm ϵ_{max} = 1.74×10^5 L/mol·cm



IR-125

λ_{max} = 795 nm ϵ_{max} = 2.04×10^5 L/mol·cm

FIG. 4a Synthesis of Estrogen-chlorin Conjugate #1 (ECC-1)

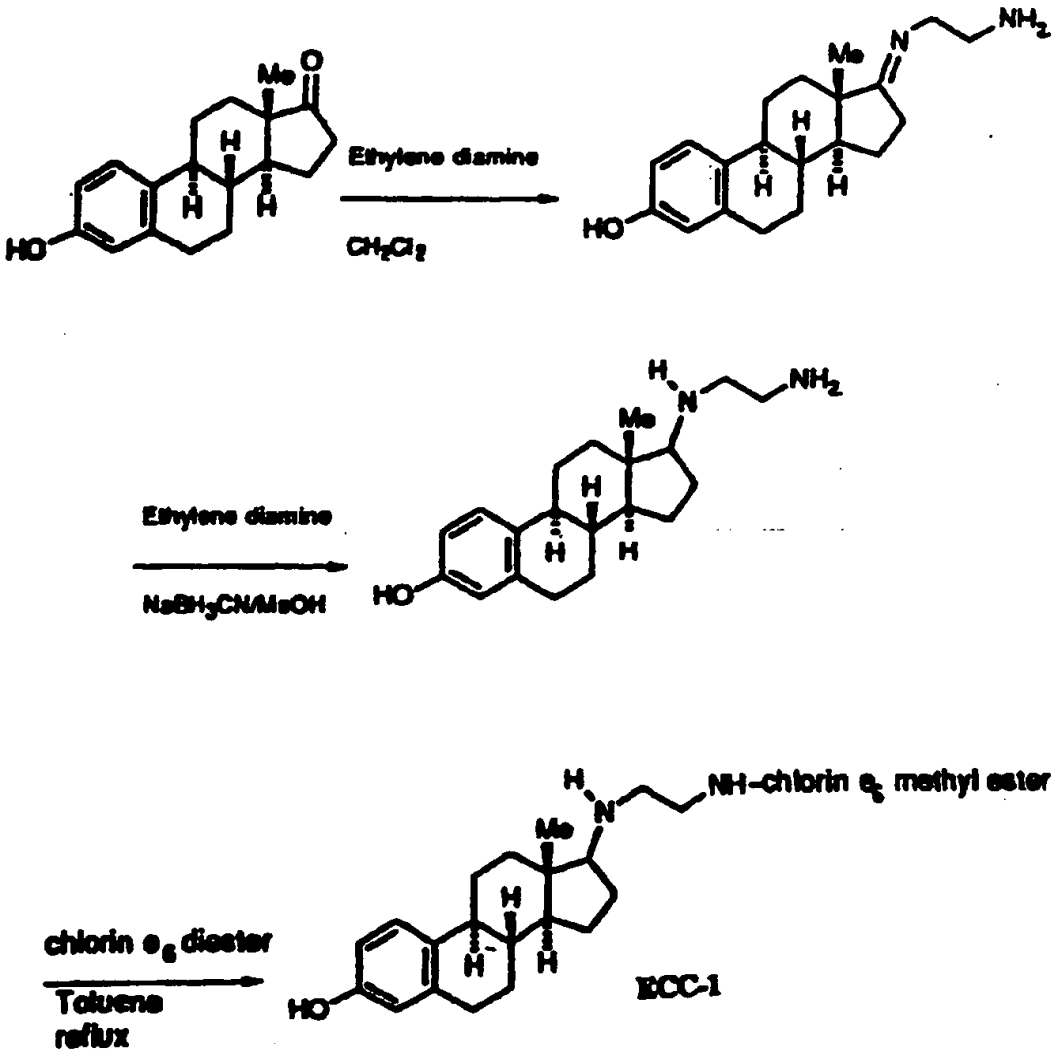
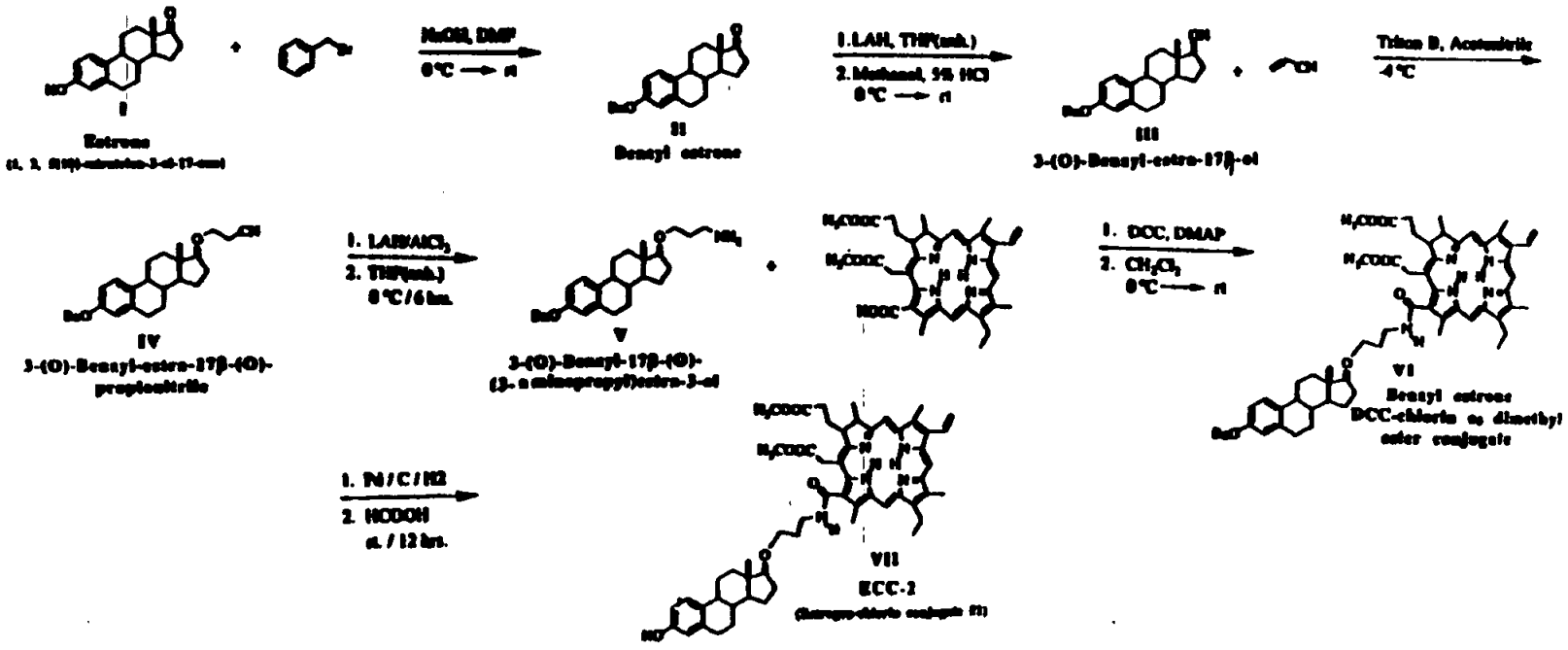


FIG. 4b

CHART I

Synthesis of Estrogen-chlorin Complex (ECC-2)

(Overall Reaction Scheme)



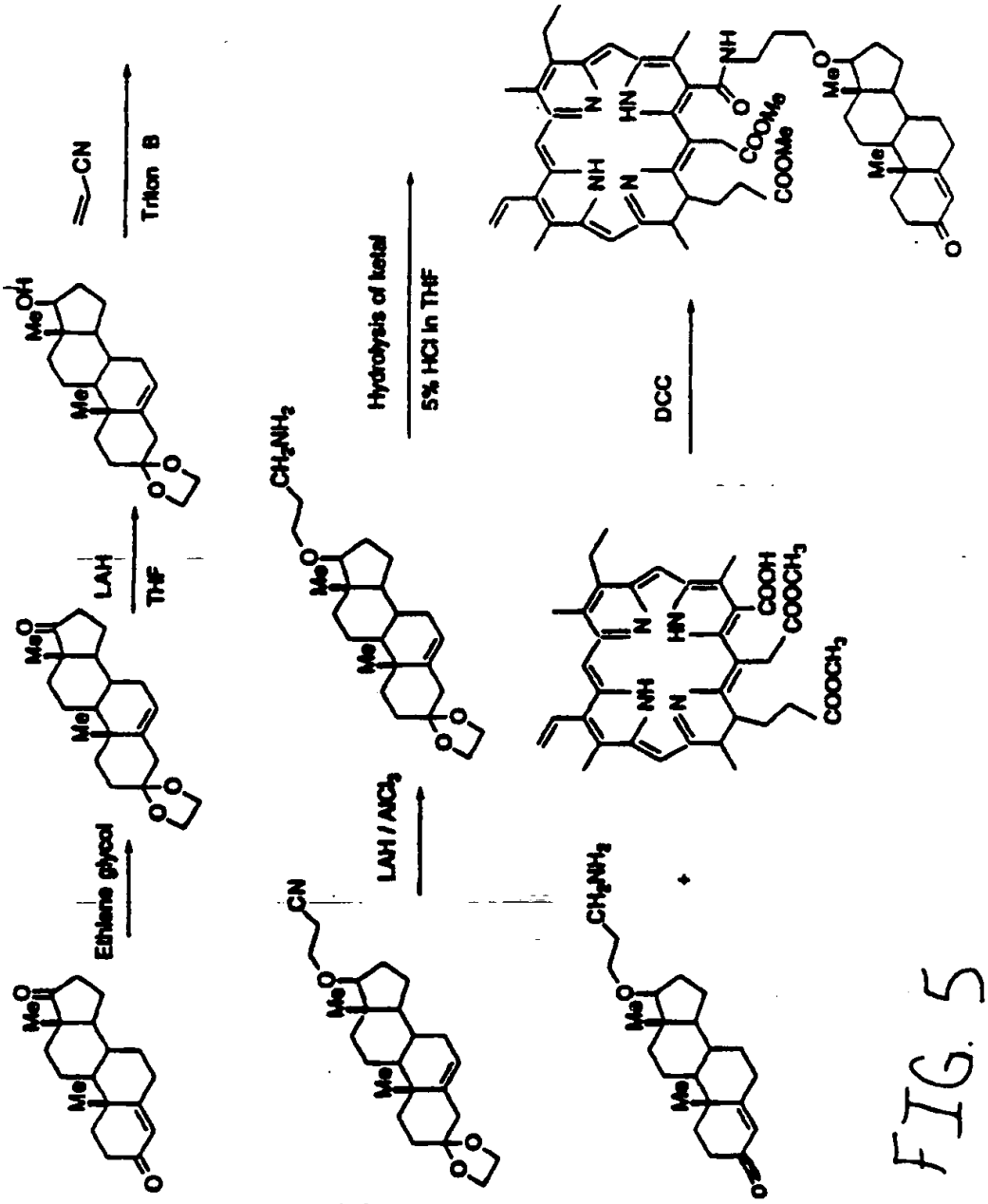
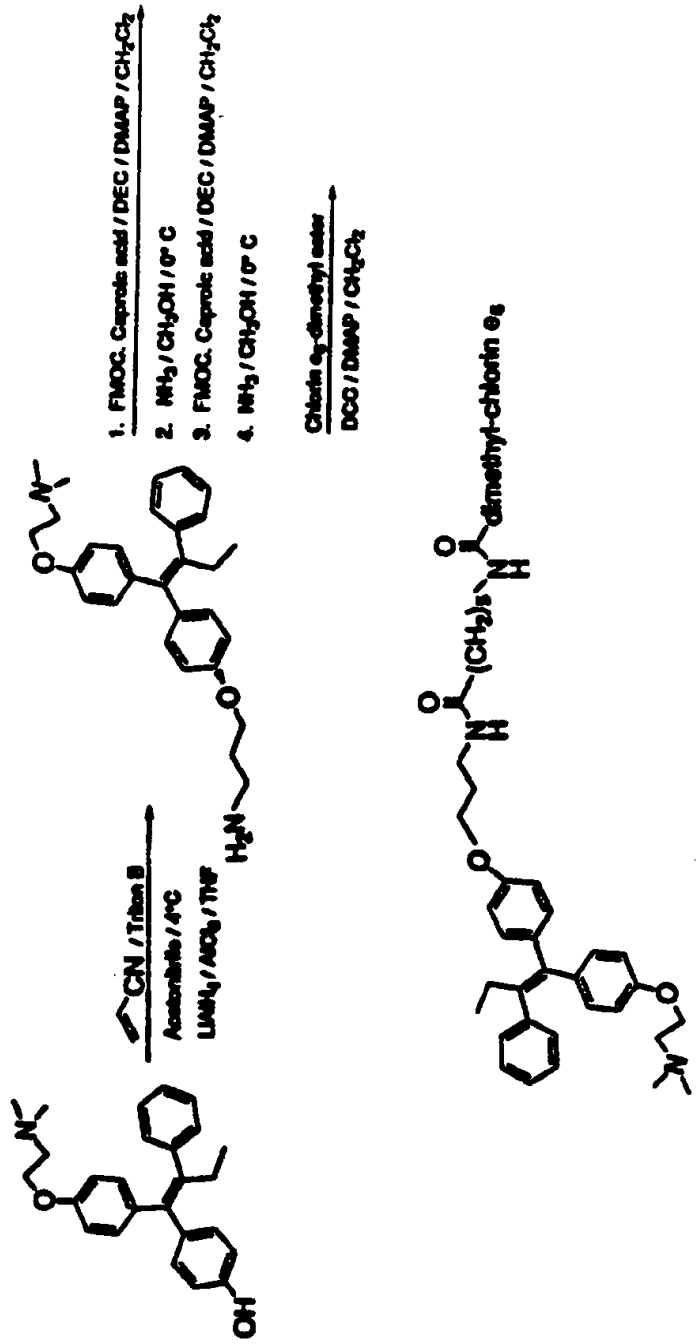

Ethlene glycol
LAH
THF
Triton B
LAH / AlCl3
Hydrolysis of ketal
5% HCl in THF
DCC

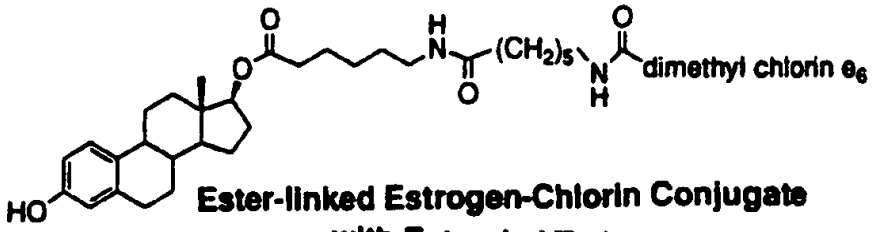
FIG. 5

Fig 5b

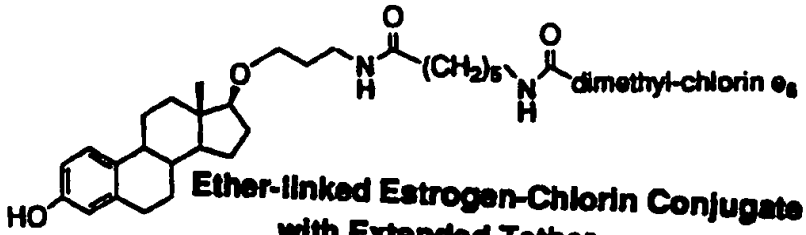


Synthesis of Tamoxifin-Chlorin-e_6 Conjugate

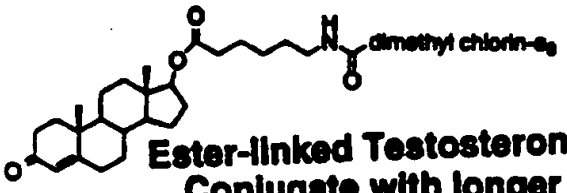
Fig 5c



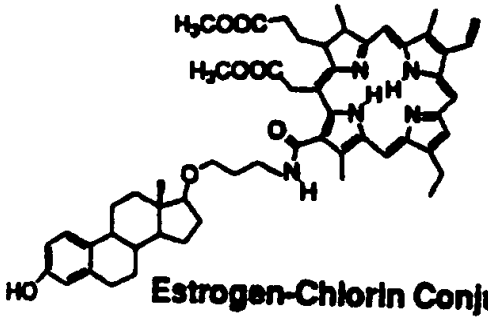
Ester-linked Estrogen-Chlorin Conjugate with Extended Tether



Ether-linked Estrogen-Chlorin Conjugate with Extended Tether

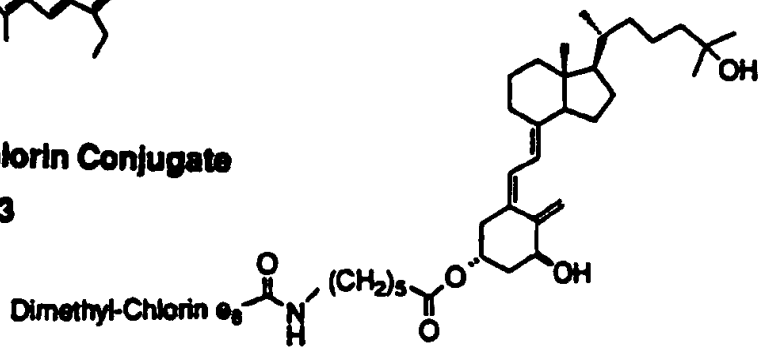


Ester-linked Testosterone-Chlorin Conjugate with longer Tether



Estrogen-Chlorin Conjugate

ECC-3



11

Vitamin D Chlorin Conjugate

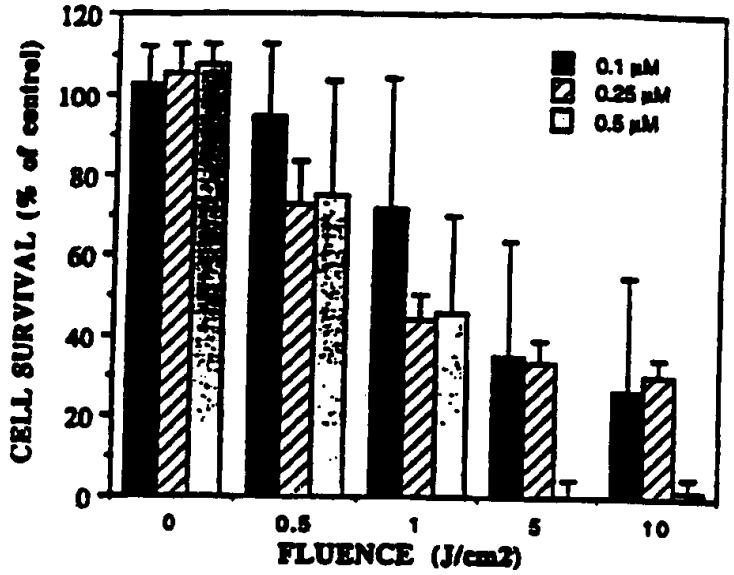

CELL SURVIVAL (% of control)
120
100
80
60
40
20
0
0.1 μM
0.25 μM
0.5 μM
0
0.5
1
5
10
FLUENCE (J/cm2)

FIG. 6a

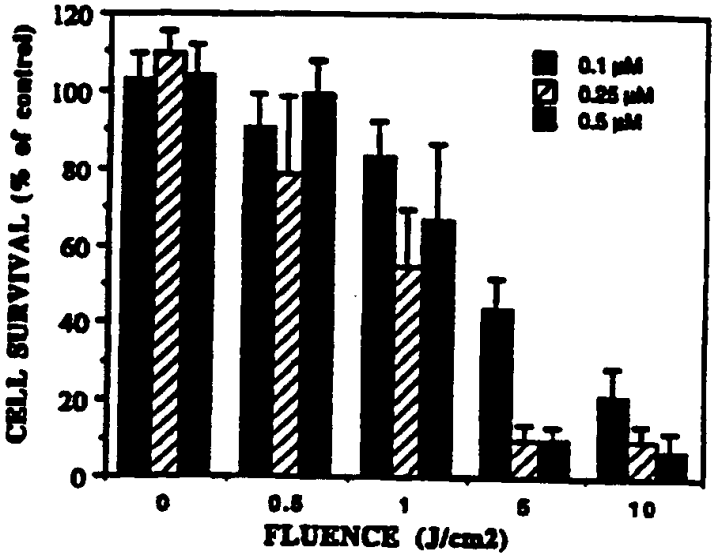

CELL SURVIVAL (% of control)
120
100
80
60
40
20
0
0.1 μM
0.25 μM
0.5 μM
0
0.5
1
5
10
FLUENCE (J/cm2)

FIG. 6b

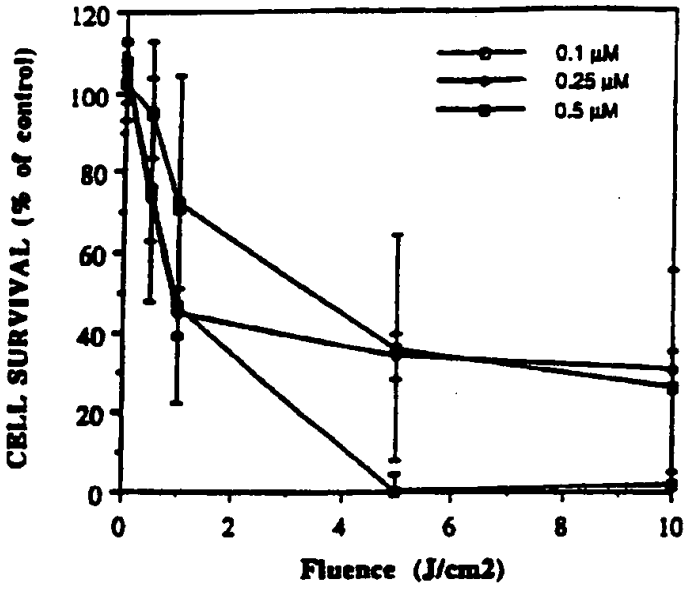

CELL SURVIVAL (% of control)
120
100
80
60
40
20
0
0
2
4
6
8
10
Fluence (J/cm2)
0.1 µM
0.25 µM
0.5 µM

FIG. 7a

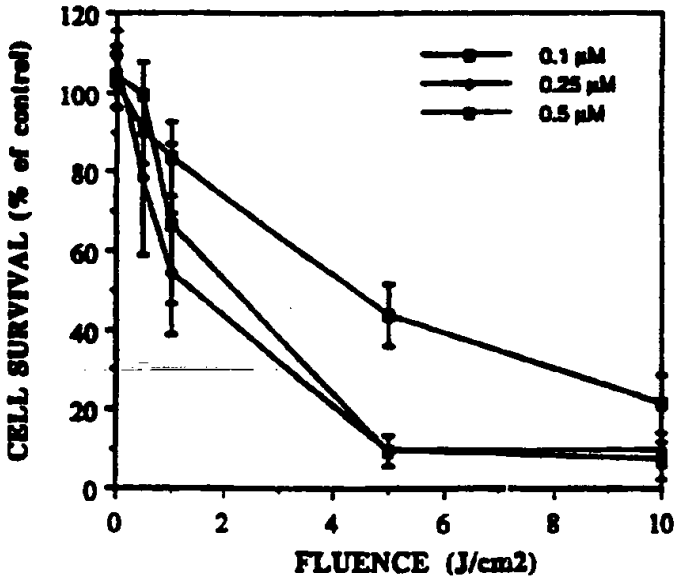

CELL SURVIVAL (% of control)
120
100
80
60
40
20
0
0
2
4
6
8
10
FLUENCE (J/cm2)
0.1 µM
0.25 µM
0.5 µM

FIG. 7b

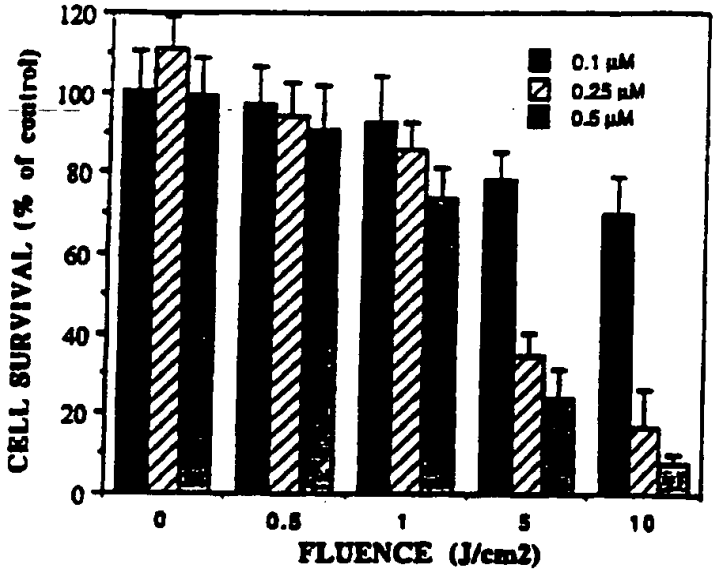

120
100
80
60
40
20
0
CELL SURVIVAL (% of control)
0.1 μM
0.25 μM
0.5 μM
0
0.5
1
5
10
FLUENCE (J/cm2)

FIG. 8a

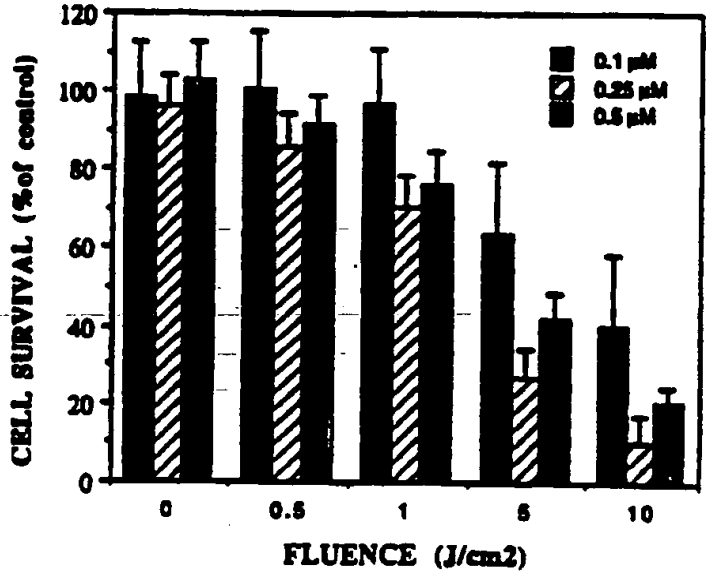

120
100
80
60
40
20
0
CELL SURVIVAL (%of control)
0.1 μM
0.25 μM
0.5 μM
0
0.5
1
5
10
FLUENCE (J/cm2)

FIG. 8b

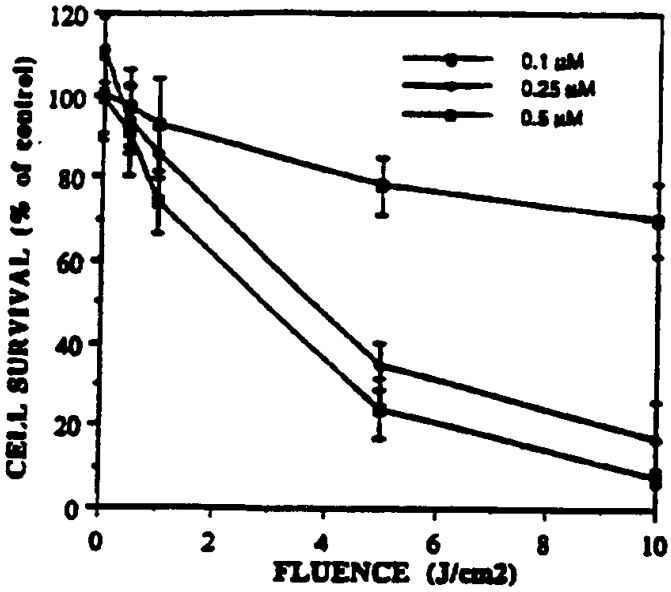

120
100
80
60
40
20
0
0
2
4
6
8
10
CELL SURVIVAL (% of control)
FLUENCE (J/cm2)
0.1 μM
0.25 μM
0.5 μM

FIG. 9a

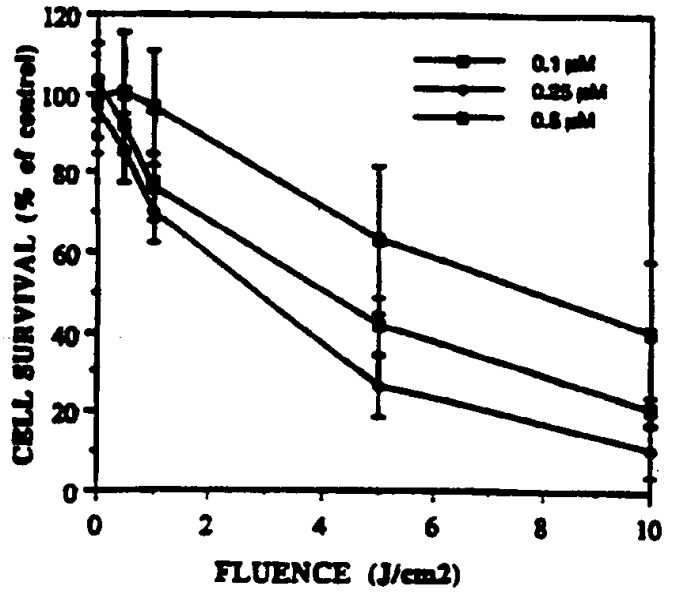

120
100
80
60
40
20
0
0
2
4
6
8
10
CELL SURVIVAL (% of control)
FLUENCE (J/cm2)
0.1 μM
0.25 μM
0.5 μM

FIG. 9b

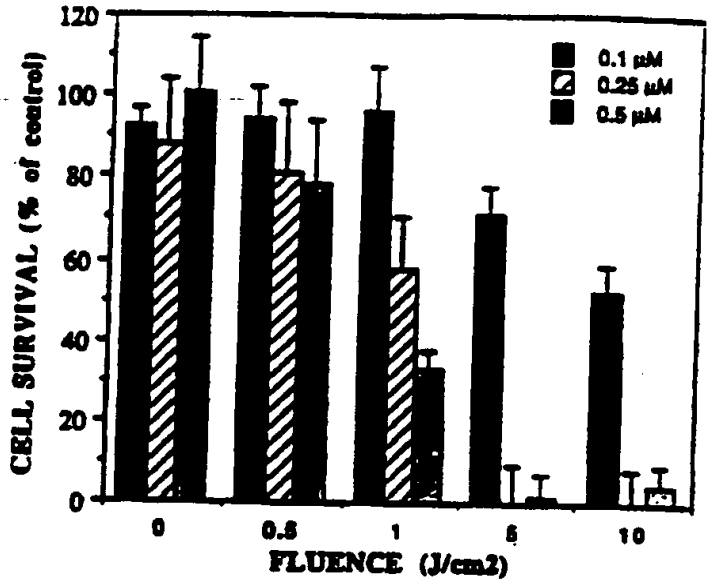


FIG. 10a

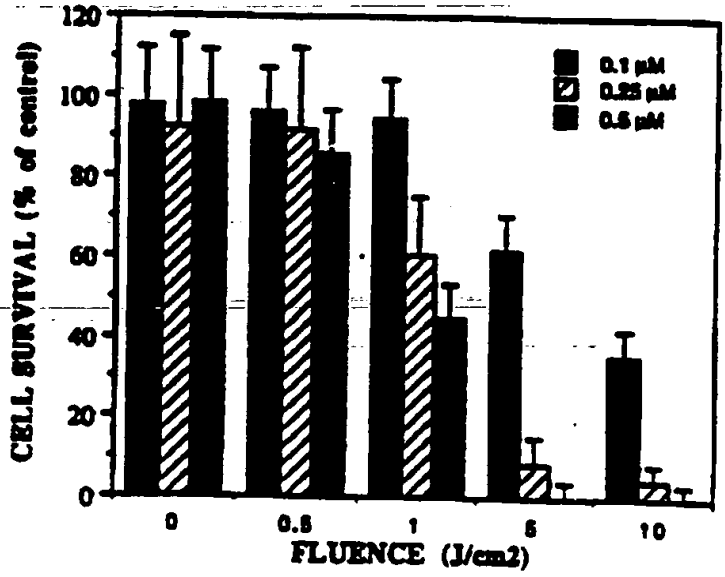


FIG 10b

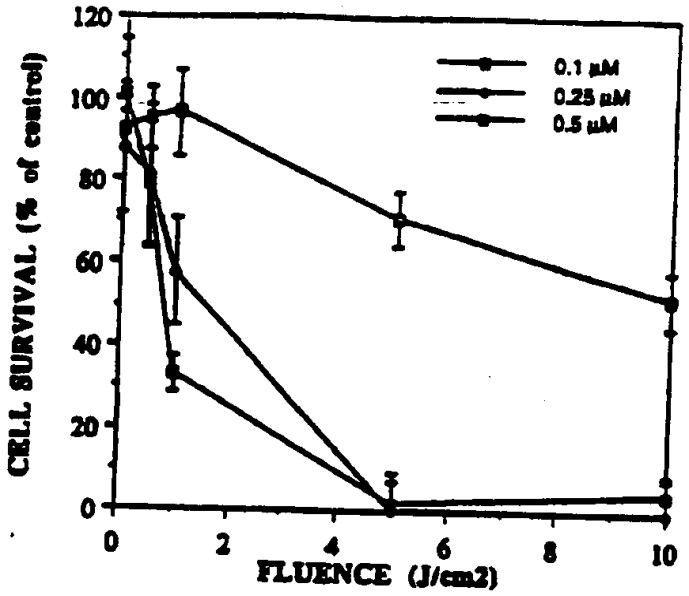


FIG. 11a

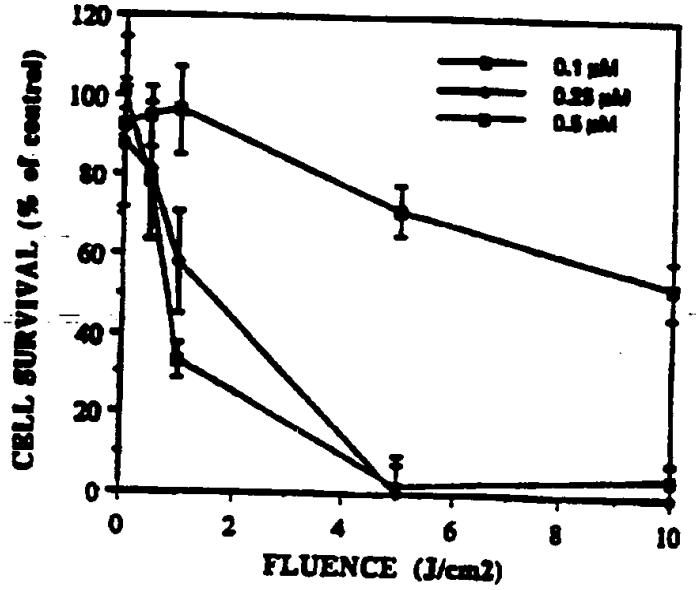
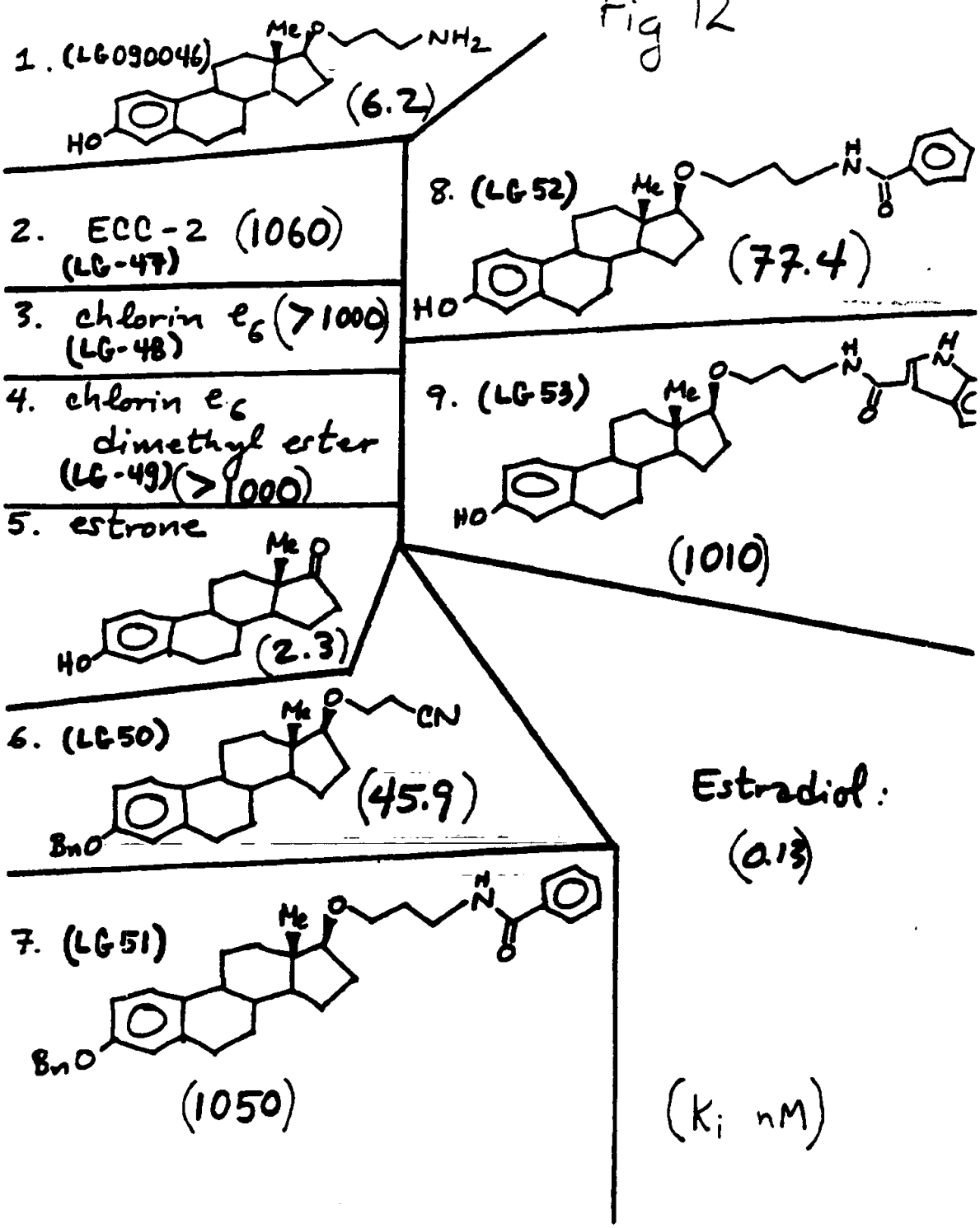


FIG. 11b

Fig 12


1. (LG090046)
(6.2)
2. ECC-2 (1060)
(LG-47)
3. chlorin e_6 (>1000)
(LG-48)
4. chlorin e_6 dimethyl ester
(LG-49) (>1000)
5. estrone
(2.3)
6. (LG50)
(45.9)
7. (LG51)
(1050)
8. (LG52)
(77.4)
9. (LG53)
(1010)
Estradiol:
(0.13)
(K_i nM)